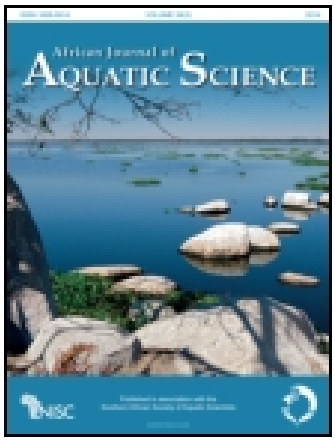

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Most endemic freshwater fish species of the Cape Floristic Region are listed as threatened, due mainly to the impacts of invasive alien fish species. The piscicide rotenone has been identified as a potential tool to aid the conservation of indigenous species through the removal of invasive fish. Rotenone was used in the Rondegat River, Cederberg, where smallmouth bass *Micropterus dolomieu* had extirpated the indigenous fish. An initial rotenone treatment in March 2012 was followed by another in March 2013. Due to concerns following the first treatment about possible build-up of rotenone between treatment stations, the second treatment included monitoring of rotenone concentrations during the treatment. Measured concentrations were consistently below the selected treatment concentration of 37.5 µg l^{-1} and dropped to below the tested effective piscicidal concentration of 12.5 µg l^{-1} at some sampling points. There was no build-up of rotenone within the treatment zones, but rotenone took longer than expected to clear out of the treatment area. The rotenone was effectively neutralised when the neutralisation station was operational, but was still detectable after neutralisation was terminated.

Keywords: freshwater fish, invasive fish, high-performance liquid chromatography, *Micropterus dolomieu*, piscicide

Introduction

The Cape Floristic Region (CFR) is one of the six aquatic ecoregions of Southern Africa (Skelton 2001) and its endemic indigenous freshwater fish fauna is highly threatened (Linder et al. 2010). Invasive alien fishes are the primary threat to indigenous fish species and for this reason management of these species is a conservation priority (Weyl et al. 2014). The piscicide rotenone has a long history of use in fisheries management (Lennon et al. 1970) and has been used successfully for biodiversity restoration (Chadderton et al. 2003; Pham et al. 2013) through the removal of alien invasive fish to facilitate the recovery of native species (Weyl et al. 2014). In South Africa, the experimental use of rotenone has been approved for use in four CFR rivers, following a comprehensive environmental impact assessment, and the Rondegat River was selected for a pilot study (Marr et al. 2012). In this river, rotenone was used to remove alien smallmouth bass *Micropterus dolomieu* (Lacepéde, 1802) that were impacting negatively on the resident indigenous fish community below a waterfall barrier (Weyl et al. 2013).

Rotenone, a botanical compound found *Lonchocarpus* and *Derris* (Leguminsae) (Brooks and Price 1961; Meadows 1973), is a highly specific toxin that affects cellular aerobic respiration, blocking mitochondrial electron transport by inhibiting NADH-ubiquinone reductase (Singer and Ramsay 1994). Besides having a strong piscicidal effect, in the aquatic environment rotenone poses a risk to a variety of gill-respiring organisms including tadpoles and macroinvertebrates (Blakely et al. 2005; Finlayson et al. 2009; Billman et al. 2011). Rotenone is a very unstable compound and degrades by a variety of mechanisms including hydrolysis ($t_{½}$-values vary from 2.0 d at pH 9 to 12.6 d at pH 5 [Thomas 1983]), photolysis ($t_{½}$ = 1.4 h [Spare 1982] to 8.2 h [Draper 2002]), and is metabolised by bacteria (Spare 1982), fish (Gingerich and Rach 1985) and mammals (Eiseman 1984). While being non-persistent, the environmental half-life of rotenone in surface water can therefore vary substantially, depending on water quality parameters and ambient conditions (Augustijn-Beckers et al. 1994; Ling 2003).

The Rondegat River was subjected to an initial rotenone treatment in February 2012 using the commercial piscicide formulation CFT Legumine (Prentiss, Spartanburg, USA) with 5% active rotenone, and using standard operating procedures (SOPs) for the use of rotenone for fisheries management (Finlayson et al. 2010). The treatment concentration was selected based on a bioassay conducted using the target species (*M. dolomieu*), water from the Rondegat River and rotenone from the stock that was later used to treat the river, as prescribed by Finlayson et al (2010). The minimum effective dose (MED) tested was 12.5 µg l^{-1} active rotenone, which resulted in 100% mortality of the treated fish (Jordaan and Weyl 2013). When eradication of fish is the objective, it is standard

practice to treat at concentrations higher than the MED, but not higher than the concentrations recommended on the label (Finlayson et al. 2010). Based on the results of the bioassay and the dosage requirements for CFT Legumine, the Rondegat River was initially treated at a rotenone concentration of 50 µg l^{-1} (i.e. four times the MED) for 6 h (Jordaan and Weyl 2013). This treatment reduced fish numbers in the treated river section to below detectable levels (Weyl et al. 2013) and had a significant short-term impact on macroinvertebrate diversity and abundance (Woodford et al. 2013).

In accordance with the SOP, a second treatment of the river was scheduled for one year after the initial treatment. Finlayson and Steinkjer (2012) recommended, for future treatments, that the number of drip stations be reduced from seven to four, spaced at equal water travel-time distances, and that the rotenone treatment rate be reduced from 50 to 37.5 µg l^{-1}. This was in response to concerns over the possible build-up of rotenone in the system and the long (more than 48 h) clearing time of the treatment section. While reducing the treatment concentration and the number of drip stations may prove beneficial to non-target biota in the river, there is an associated risk of not maintaining an effective piscicidal concentration throughout the treatment. The treatment was therefore scheduled to include the monitoring of active rotenone concentrations at selected points in the river at various times during the treatment. This included sampling rotenone concentrations below a neutralisation station at the end of the last treatment zone, where the active rotenone was neutralised using a 2.5% solution of potassium permanganate (Finlayson et al. 2010).

The aims of the study were to determine whether the rotenone was maintained at piscicidal concentrations throughout the treatment period, whether there was an accumulation of rotenone between treatment stations resulting in concentrations higher than the treatment concentration, and whether all active rotenone had been either neutralised or cleared from the system 48 h after initiating the treatment.

Materials and methods

Treatment area

The treatment area consisted of the lower section of the Rondegat River between a waterfall barrier (32°16.657′ S, 18°58.596′ E) and a neutralisation point (32°15.365′ S, 18°57.135′ E) close to Clanwilliam Dam (Figure 1). Four rotenone drip stations were placed in the treated river section at roughly equal distances apart constituting four treatment zones. On 13 March 2013 the river was treated with the commercial piscicide formulation CFT Legumine (US EPA Registration number 75338-2) containing 5% active rotenone, at a concentration of 0.75 ppm. This treatment concentration contains 37.5 µg l^{-1} active rotenone and should ensure that a piscicidal concentration of >12.5 µg l^{-1} is maintained throughout the six-hour treatment duration from 09:00 to 15:00. A neutralisation station containing potassium permanganate ($KMnO_4$) was placed as the end of the treatment area and administered potassium permanganate at a concentration of 25 mg l^{-1} for approximately 18 h. *Micropterus dolomieu* 'sentinel' fish were deployed in keep nets at the end of each treatment zone and below the neutralisation station, after Finlayson et al. (2010), to determine the efficacy of the treatment.

Water quality parameters were measured with a Hanna HI98129 Combo pH and electrical conductivity meter, and turbidity (NTU) was measured using a Hanna HI 98703 turbidimeter (HANNA Instruments Inc., Woonsocket, USA) at 14 sites in the treated river section. Mean values were: pH 7.46 (SD 0.16); temperature 22.7 °C (SD 1.2); turbidity 2.07 NTU (SD 0.97); and conductivity 52 µs s^{-1} (SD 5).

Sampling design and sample collection

Water samples were collected at 0.5 h, 2 h, 4 h and 6 h time-intervals after the start of the treatment. Control samples were collected 2 h and 4 h after initiating the treatment 5 m upstream from treatment Station 1. Samples were collected from the treatment zones at the four time-intervals stated 30 m below the drip station (start of treatment zone) and again 30 m above the next drip station (end of treatment zone). Water samples were collected at the four selected time-intervals, as well as at 8 h, 12 h, 24 h and 48 h, at the end of the treated river section (i.e. at the neutralisation station) and at 30 m below that. Water samples were collected in 500 ml amber glass bottles with Teflon-lined caps by submerging the bottles 30 cm below the water surface and filling them to capacity. The bottles were then capped, placed on ice until transfer to a refrigerator, where they were maintained at 4 °C before transfer to the laboratory at the end of the treatment. Here, all samples were decanted to laboratory-grade plastic bottles and frozen at –20 °C for two weeks until analysis. To test for possible effects of freezing and thawing on measured rotenone concentrations, control standards were prepared, analysed and then frozen, stored and reanalysed for rotenone concentration.

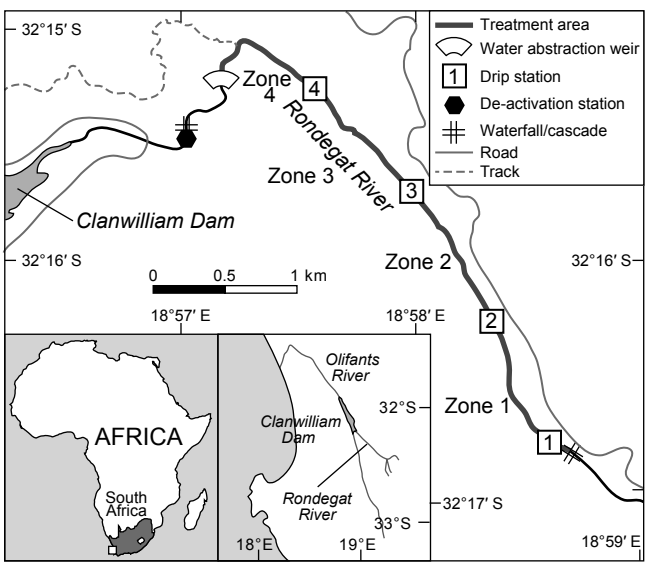

Figure 1: Map of treated section of the Rondegat River, indicating locations of rotenone drip stations and deactivation station. Treatment Zones 1–4 were the river segments between respective drip stations

Sample preparation and high-performance liquid chromatography analysis

Rotenone concentrations were determined using the method described by Dawson et al. (1983). Water samples were buffered to pH 5 with 2 ml buffer reagent (14.8 mM acetic acid, 70.4 mM sodium acetate) added to the 100 ml sample. Sep Pak C18 cartridges (Waters, Milford, USA) were preconditioned using 2 ml methanol followed by 5 ml water. The buffered water samples were filtered through the Sep Pak cartridges at 40 ml min^{-1} using a syringe. The sample was eluted using 2 ml methanol concentrating the sample 50 times. Samples of CFT Legumine were also diluted to an expected concentration of 50 µg l^{-1} and the concentration verified. Water samples were analysed against the standard and against MilliQ purified water with known concentrations of rotenone to test column retention. The rotenone high-performance liquid chromatography (HPLC) standard (Sigma-Aldrich, St Louis, USA) was dissolved and diluted in methanol and used to construct a concentration standard curve. The concentrations of the rotenone in the samples were determined from the standard curve. In addition, a rotenone standard dissolved in water and extracted were analysed to determine the test the extraction efficiency of the Sep Pak C18 cartridges. HPLC analysis was performed using a Waters Breeze HPLC with 717 Autosampler and 2487 dual wavelength UV detector (Waters, Milford, USA). The stationary phase consisted of a Phenomenex Kinetex 2.6u C18 column (150 mm × 4.6 mm) (Kinetex, Torrance, USA). The mobile phase consisted of methanol and water at a ratio of 70:30 with a flow rate of 1 ml min^{-1}. The optical density of the samples was measured every second at a wavelength of 295 nm.

Statistical analysis

Treatment zones were taken as replicates. The mean rotenone concentration at 0.5 h, 2 h, 4 h and 6 h time-intervals was determined for the start and end of each treatment zone and this was compared to the nominal treatment concentration (37.5 µg l^{-1}) and the MED (12.5 µg l^{-1}). To determine whether the rotenone had accumulated between treatment stations, the mean rotenone concentrations measured throughout the treatment time (t = 0.5 h to t = 6 h) were compared between the start and end of each zone and between zones using ANOVA. *Post hoc* analysis was done using the Tukey HSD test.

Results

The HPLC results from the pure and extracted concentration standards showed that the yield from the extraction method was 94.8% (SD 0.89). The minimum concentration standard that could be accurately measured was 0.25 µg l^{-1}. The rotenone concentration in the CFT Legumine was measured at 5.02% (SD 0.03). The HPLC results showed that freeze/thawing had no significant effect on rotenone concentration in control samples. When analysing rotenone control samples of 50 µg l^{-1}, an average of 50.3 µg l^{-1} (SD 0.2) were measured for frozen controls and 50.1 µg l^{-1} (SD 0.5) for unfrozen controls. All rotenone concentrations measured are presented in Table 1. Mean concentrations at the start of the treatment zones ranged from 24.2 µg l^{-1} (SD 10.2) to 28.7 µg l^{-1} (SD 1.5) over the six-hour treatment period and were not significantly different from each other ($p > 0.05$) (Figure 2). Concentrations at the ends of the treatment zones showed greater variation, ranging from 0.9 µg l^{-1} (SD 4.48) to 8.6 µg l^{-1} (SD 7.6). Some significant differences were observed between the mean values at the ends of the treatment zones (Figure 2). For each of the four treatment zones, a significant reduction in mean rotenone concentration was observed between the start and end of the treatment zone ($p < 0.05$) (Figure 2).

The mean treatment concentrations at the start of the treatment zones for every time-interval (t = 0.5 h to 6 h) were consistently higher than the MED, but those at the end of the treatment zones were consistently lower than the MED (Figure 3). Rotenone travel time through the different treatment zones was variable. Rotenone was first detected at the ends of treatment Zones 1–3 within 4 h travel time, but was only detected in Zone 4 at 6 h (Table 1).

Discussion

The use of standardised HPLC methodology (Dawson et al. 1983) was successful for determining rotenone concentrations. Measured rotenone concentrations during the treatment varied both temporally and spatially during the course of the treatment (Table 1). This can partly be

Table 1: Rotenone concentrations (µg l^{-1}) at sampling locations on the Rondegat River at t = 0.5 h to 6 h on 13 March 2013. Additional samples were taken at the end of treatment Zone 4 and below the neutralisation station at t = 8 h to 48 h

Sampling locality	Sampling time-interval (h)								
	0.0	0.5	2	4	6	8	12	24	48
Above treated river section (control)	–	–	0	0	–	–	–	–	–
Zone 1: start	–	18.04	16.8	27.15	28.77	–	–	–	–
Zone 1: end	–	0	3.8	14.84	18.16	–	–	–	–
Zone 2: start	–	23.3	19.29	30.78	31.94	–	–	–	–
Zone 2: end	–	0	0	8.07	10.64	–	–	–	–
Zone 3: start	–	32.44	39.23	28.43	32.23	–	–	–	–
Zone 3: end	–	0	0	8.07	5.22	–	–	–	–
Zone 4: start	–	30.09	21.48	28.51	16.21	–	–	–	–
Zone 4: end	–	0	0	0	0.43	1.74	8.89	7.04	2.94
Below neutralisation station	–	0	0	0	0	0	0	0	1.56

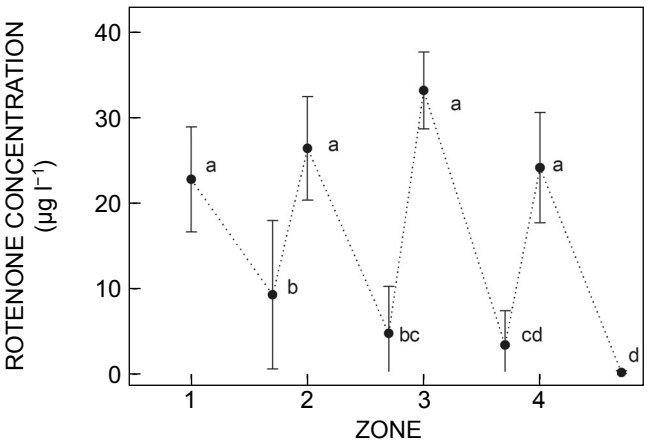

Figure 2: Mean rotenone concentrations at t = 0.5 h to 6 h at the start and end of each treatment zone during treatment of the Rondegat River on 13 March 2013. Letters above error bars denote statistically significant differences ($p < 0.05$). Error bars denote SD

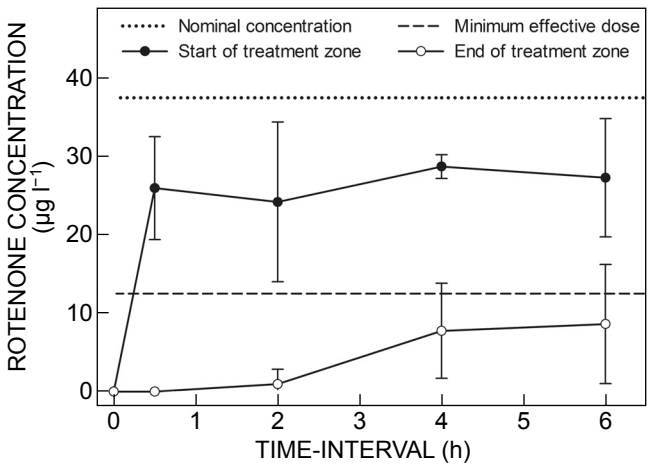

Figure 3: Mean rotenone concentrations at the start and end of treatment Zones 1–4 at time-intervals of t = 0.5 h to 6 h during treatment of the Rondegat River, relative to the nominal (applied) concentration and the minimum effective concentration. Error bars denote SD

explained by variability in instream habitat characteristics, as these would have affected the amount of rotenone present at any time and point in the river. Much of the Rondegat River consists of shallow, interconnected pools, mainly with rock and cobble substrate, interspersed with riffle and run habitats (Weyl et al. 2013). These areas have a relatively constant flow of 0.127 cm^3 s^{-1} (SD 0.038), and the impact of habitat on rotenone travel time in the water is expected to be minimal. In some areas, mainly towards the lower end of the treated river section and within treatment Zone 4, there are large and relatively deep pools, which have a slow volume turnover. The rotenone travel time in these areas is substantially slower than in riffle, pool and run habitat. Evidence for this is that rotenone was below detectable limits in this zone until 6 h after commencement, despite the piscicide concentrations being maintained throughout the treatment (t = 0.5 h to t = 6 h) at the start of this treatment zone (Table 1). Rotenone was also still present at the end of treatment Zone 4 for 48 h after the treatment was initiated, indicating the slow clearing time of the rotenone through the treated river section. In contrast, rotenone travel time in treatment Zone 1 was relatively rapid and rotenone was detected at the end of this zone two hours into the treatment (Table 1). Travel time was slower in Zones 2 and 3, with the first rotenone detected at the end of these zones after 4 h, again probably a result of the impact of instream habitat on flow and thus on rotenone movement in the river.

The mean treatment concentration measured at the start of the treatment zones did not exceed the nominal concentration of 37.5 µg l^{-1} (Figure 3). At no time during the treatment period was this concentration exceeded, except at the sample taken two hours into the treatment in treatment Zone 3, where a concentration of 39.2 µg l^{-1} was recorded (Table 1). The consistently lower concentrations measured during the treatment may be attributed to inconsistent application rates, uneven mixing in the water column, a high biological demand for rotenone in the river, or a breakdown of rotenone during the sample storage phase prior to analysis. The latter is unlikely as all samples were transported back to the laboratory within 24 h and were then stored in the dark at –20 °C until analysis. Rotenone breakdown during the treatment is more likely, due to ambient environmental conditions during the treatment. The degradation of rotenone in aquatic environments is influenced by temperature, water volume, pH, surface area, substrate, sunlight, turbidity and dissolved oxygen (Bettoli and Maceina 1996). Phytoplankton, zooplankton and bacteria are also likely to influence the rate at which rotenone disappears from water, and these are likely to be most active in warm water (Gilderhus et al. 1988). Rotenone decays under first order kinetics and, in waters with neutral pH or above, the half-life of rotenone would vary from 0.6 d to 7.7 d, depending on water temperature and depth (Finlayson et al. 2001).

Rotenone degrades rapidly at temperatures >20 °C and slowly at temperatures below 10 °C (Engstrom-Heg and Colesante 1979; Gilderhus et al. 1988). Strong UV radiation has been shown to influence the rate of rotenone breakdown significantly (Gilderhus et al. 1988) and a photolysis half-life of 1.4 h (Spare 1982) to 8.2 h (Draper 2002) has been reported for rotenone. High-intensity UV radiation is typical of the Cederberg in summer (Janse van Rensburg 2009), and water temperatures during the treatment averaged in excess of 20 °C. It is likely that these two factors influenced the active rotenone concentration during treatment and could have resulted in rapid rotenone breakdown upon application, resulting in measured concentrations lower than the treatment concentration and, in some cases, lower than the lowest tested piscicidal concentration of 12.5 µg l^{-1} (Jordaan and Weyl 2013). The latter is a potential source of concern as it could have enabled the survival of some *M. dolomieu* individuals following the treatment. However, as toxicity is a function of exposure concentration and exposure time, the lower concentrations should have been compensated for by the longer exposure time as a result of the slow movement of the rotenone in the

lower half of the treated river section. This is supported by the complete mortality of all sentinel fish (*M. dolomieu*) that had been deployed at the end of each treatment zone.

There is no evidence that the rotenone accumulated between drip stations. In fact, rotenone concentrations decreased significantly between the start and end of each treatment zone, and the extent of this reduction increased with distance downstream (Figure 2, Table 1), indicating faster breakdown or a greater biological demand from the river, perhaps attributable to different land-use patterns such as alien vegetation clearing resulting in more direct sunlight penetration into the water column. There was also an increase in cattle grazing near the bank of the river, plus some fruit orchards further downstream, which could have contributed nutrients, thereby affecting water quality characteristics and thus the biological demand.

The rotenone had a slow clearing time through the treated river section, probably as a result of the slow travel time through treatment Zone 4, as low concentrations of rotenone were detected in the river 48 h after initiation of the treatment (Table 1). The delay may have been caused by one or more of the following factors: (1) pockets of rotenone-containing interstitial water in the riverbed (i.e., in aquatic palm roots); (2) the backpack-spraying of seeps, semi-connected backwater areas and furrows with a more concentrated rotenone solution (1% to 2% CFT Legumine); and (3) physically connected backwater areas. Rotenone was effectively neutralised by the potassium permanganate drip until 24 h, whereafter neutralisation was terminated. Rotenone was detected below the neutralisation point at 48 h, indicating that the 24 h neutralisation time was inadequate. Complete inactivation would have required the neutralisation station to run for longer than 48 h. In the case of the Rondegat River, the presence of active rotenone more than 24 h after the treatment had been terminated is of no concern, as the river runs into Clanwilliam Dam which would have diluted the rotenone to negligible concentrations. Furthermore, the 37.5 µg l^{-1} rotenone concentration was within the range recommended for eradiating sensitive species and to minimise impacts to non-target aquatic invertebrates (Finlayson et al. 2009). Effective neutralisation will, however, be critical for rotenone operations where this is not the case and where there may be impacts on non-target downstream biota such as aquatic invertebrates and amphibians.

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