Determination of human and veterinary antibiotics in indirect potable reuse systems

Francesco Busetti[†] and Anna Heitz

CWQRC, Curtin Water Quality Research Centre, Department of Applied Chemistry, Curtin University of Technology, GPO Box U1987, Perth, Western Australia 6845, Australia

[†]Corresponding author. E-mail: f.busetti@exchange.curtin.edu.au

Fax: +61 8 9266 3547

Abstract

This paper reports the optimization and validation of an analytical method for the determination of the residual concentration of the prescription antibiotics metronidazole, trimethoprim, sulfamethoxazole, azithromycin, clindamicyn, clarithromycin, erythromycin-H_2O, roxithromycin and tylosin in wastewater and advanced treated wastewater. The method applied was used in a study of removal efficiency of these compounds at a full scale operational water reclamation plant using microfiltration-reverse osmosis (MF-RO) (Kwinana, Western Australia). The analytical procedure involves off-line solid-phase extraction (SPE) followed by liquid chromatography - tandem mass spectrometry (LC-MS/MS) operated in multiple reaction monitoring mode. Method validation included determination of linearity, accuracy, precision, method limits of quantitation (MLQs), reproducibility and matrix effect. SPE recoveries were generally higher than 89% for both pre- and post- RO water, except for erythromycin which yielded approximately 50% recovery. The overall precision of the method was better than 16% RSD (relative standard deviation), for all compounds and matrices. MLQ ranged between 23–53 ng/L and 2.5–31 ng/L for pre- and post- RO water, respectively. In-house reproducibility expressed as RSD was generally better than 10%. Inter-laboratory tests revealed a generally good agreement between concentrations of antibiotics reported by all participants. Results demonstrate that MF/RO treatment is capable of removing antibiotics present at relevant environmental concentration in secondary effluent (from the low to-mid ng/L range) to below MLQs (2.5–31 ng/L), and more importantly, three to six orders of magnitude below the health guideline values developed for this project. Estimated RO rejections ranged between > 91 and 99%.

Keywords: water reuse; indirect potable reuse systems; reverse osmosis; PPCPs; antibiotics; LC-MS; SPE; wastewater.

1. Introduction

To cope with water shortage, reuse of effluents for direct or indirect potable purposes is increasingly becoming a necessity in many countries around the world [1, 2]. To date, the majority of the secondary treated wastewater produced globally is discharged into the environment (i.e. rivers, lakes, oceans) while only a small portion is reused. Currently, recycled water derived from secondary treated wastewater is mainly used for beneficial purposes other than augmentation of drinking water supplies. These applications include agricultural and landscape irrigation, industrial processes and toilet flushing; indirect potable reuse applications include replenishing of natural sources through Ground Water Recharge (GWR), through Soil Aquifer Recharge (SAT) or through River Bank Filtration (RBF) [2].

Examples of successful water recycling projects to augment drinking water supplies are Orange County Water District in California [3] and Singapore NEWater [4] which are continuously promoting innovative programs to turn wastewater into high quality recycled water. The health and environmental impacts of trace chemicals is a key issue in recycling treated wastewater for direct/ indirect potable reuse. Lack of knowledge of health and environmental risks associated with the presence of "chemicals of concern" (COCs) and their removal by advanced treatment processes, have, to some extent, hindered the establishment of large reuse schemes in Australia; even with the advent of advanced technological treatments such as MF/RO, indirect potable reuse (IPR) has been slow to gain public acceptance [5]. Gaining public trust in organisations, as well as in the technology, is of vital importance. Scientific studies to evaluate treatment processes, serve to build the body of knowledge that is required for regulators and decision makers and may eventually lead to increased public acceptance of IPR [5].

To ensure consistently high quality recycled water, a multiple barrier approach is generally adopted in water reclamation plants [1, 2, 6]. Barriers can comprise a variety of possible processes, including: "source protection" which aims to minimise/ avoid COCs entering the wastewater; conventional wastewater treatment, which uses denitrification, nitrification and activated sludge treatment to remove suspended solids and nutrients from the influent wastewater, but often is not an effective barrier to COCs; microfiltration/ultrafiltration (pre-treatment) to remove residual suspended

solids, colloidal particles and micro-organisms; physical processes such as nanofiltration (NF) or reverse osmosis (RO) which provide "tighter filtration" or "molecular sieving" for removal of COCs, residual nutrients, salts, micro-organisms and viruses; UV disinfection (to ensure that all micro-organisms and viruses are inactivated); other chemical treatments such as advanced oxidation processes (AOPs) to remove residual COCs. AOPs rely on hydroxyl radicals which are powerful non-selective oxidizing agents and, in principle, are able to mineralize the residual organic carbon into CO_2 and H_2O. However, because of economic and energy restrictions, AOP processes at dosages resulting in complete mineralization of target compounds are often not sustainable [7]. In addition, several compounds are not amendable to an oxidative attack. Incomplete oxidation of PPCPs may result in the formation of oxidation by-products with reduced pharmacological effect compared to the parent drug compound. For example, it has been demonstrated that it is possible to reduce the estrogenic activity of 17α-ethinylestradiol significantly using O_3 and other oxidants [7]. Other studies have shown that in nearly all cases elimination of antibacterial activity has been achieved following treatment of a number of anti-infective prescription drugs with O_3 [8]. The chemical structures and toxicological and pharmacological properties of degradation by-products arising from advanced oxidation processes are largely unknown for most pharmaceuticals and personal care products (PPCPs) [9, 10] although research in this area is developing (e.g. [7, 11]).

Regardless of the extent of tertiary treatment applied to "polish" or "refine" the product water, public concern regarding wastewater reuse for indirect potable reuse is mainly linked to the residual concentration of virus and inorganic and organic micropollutants in the recycled water [1, 2, 6]. The effective removal of such organic contaminants by tertiary treatments has been a major challenge in the establishment of large water reuse schemes. Pollutants in recycled waters that pose major health concerns include heavy metals, organic compounds with suspected carcinogenic properties (e.g. nitrosamines and halogenated disinfection by-products), unregulated pharmaceuticals and personal care products (e.g. endocrine disrupting compounds, cytostatics and antibiotics) and other unregulated trace organic compounds (i.e. plasticizers, surfactants) derived from both domestic and industrial activities [1, 2, 12, 13]. In the last decade, regulatory agencies have struggled to deal with these wastewater-derived contaminants because of (1) their characteristic low residual concentrations in secondary/tertiary effluents, (2) a lack of standardized analytical

protocols and (3) a lack of knowledge of potential health and environmental risks associated with their presence [6].

The work presented in this paper is part of a larger project to investigate the effectiveness of advanced tertiary treatment processes, particularly MF/RO, to treat secondary treated wastewater for indirect potable reuse. The city of Perth in Western Australia (WA) is facing a future of compromised water supplies and a major initiative of the Western Australian State Water Strategy is 30% wastewater reuse by 2030. Recharge of tertiary treated wastewater to aquifers beneath the Swan Coastal Plain including Perth's major drinking water aquifer, the Gnangara Mound, and re-extracting that water as source of drinking water, will be an important component of achieving this goal. Characterization of contaminants in wastewater pre- and post-advanced treatment and an assessment of the health and environmental risks are necessary to refine health and environmental regulations and to inform the community about the recycled water quality [14, 15].

The main objectives of this study were: (1) Develop and validate a multi-component analytical method for analyses of nitroimidazole, sulfonamide, lincosamide and macrolide antibiotics (Table 1) in secondary effluent and post- RO treated water based on LC-MS/MS. (2) Provide information on the occurrence of target analytes in secondary effluents from WWTPs in Western Australia. (3) Assess the efficacy of MF/RO to remove specific antibiotics (4) Provide the first locally relevant information to assess whether recycled water is safe under the proposed treatment and under Western Australian conditions.

Antibiotics and, in general, PPCPs are not yet considered in drinking water quality guidelines in Australia [16] or elsewhere [17], although they are included in recently published Australian guidelines for recycled water [18]. Antibiotics are known to persist during conventional biological treatment processes and consequently many of them have been detected in secondary effluents at measurable concentration levels (i.e. [1, 6, 19-28]). Moreover, tertiary treatments (e.g. MF/RO) are not 100% efficient in removing organic contaminants [1, 6, 14, 15]. Therefore a robust analytical protocol and a comprehensive monitoring plan is required to assess their presence in water produced for direct and indirect potable reuse, and to investigate their removal during tertiary treatment.

2. Experimental

2.1. Sampling and sample pre-treatment

Secondary treated wastewater samples were collected from Beenyup and Woodman Point WWTPs which are located respectively on the north-west and south-west of the city of Perth, WA. Both WWTPs employ classical biological treatment for removal of suspended solids, nutrients and phosphorous from raw influent wastewater. A large portion of the secondary effluent from Perth's WWTPs is currently being discharged into the Indian Ocean (total volume of secondary effluent produced in 2008 was 124 GL). Recently, a small portion of secondary effluent from Beenyup and Woodman Point WWTPs has been fed into two water recycling facilities, where secondary treated wastewater is further treated using MF/RO. Full details of the characteristics of the Kwinana Water Reclamation Plant (KWRP) are provided in Busetti *et al.* [29]. A flowchart of KWRP indicating the sampling points is shown in Figure 1, available as supplementary information. The water produced (approximately 17 ML/day for KWRP) is re-used as general process water by neighbouring industrial facilities, reducing Perth's total demand for scheme water by about 2%. Operational conditions at KWRP were: Dow Filmtec BW30-400-FR RO membrane (composite polyamide, 8 inch, spiral wound); recovery: 70%; permeate flux: 18 L/m^{2}h; pH feed: 6.1; chemical dosing for chloramination (ammonia, hypochlorite) and pH control (sulphuric acid) occur prior to microfiltration.

The second wastewater facility investigated in this work is located at Beenyup, north-west of Perth. This pilot plant comprises the first stage of a larger project investigating indirect potable reuse through groundwater replenishment. The pilot plant receives approximately 100 kL/day of secondary effluent for MF/RO treatment. Operational conditions at Beenyup were: Hydranautics ESPA-2 4040 RO membrane (composite polyamide, 4 inch, spiral wound); recovery: 70%; permeate flux: 19.7 L/m^{2}h; pH feed: 6.5; chloramination and pH control procedures are identical to those at the KWRP facility (see above).

Composite and grab samples were collected pre- and post- RO treatment from Woodman Point/KWRP plant on three days over a week-long period 30 May - 7 June 2007. Twenty-four hour composite samples were taken using an automated ISCO

4700 refrigerated sampler while grab samples were collected from the relevant stream at the time of sampling. Aqueous samples taken from Beenyup WWTP and Beenyup RO Pilot plant were used to test the reproducibility of the analytical method and sampling procedure. Further details are given in Section 3.3.4. Field and trip blanks were also collected on each day of sampling. Samples were preserved with 100 mg/L of NaN_3, which was added as a solid to the amber glass sample bottles (4 L) prior to sampling. Samples were stored at 4 °C until sample extraction, which was typically performed within 2–3 weeks of sampling.

2.2. Inter-laboratory trial

The aqueous samples selected for the inter-laboratory trial included a groundwater sample, five secondary treated wastewaters, and three post- RO treated waters collected from various locations around the city of Perth. On arrival at the CWQRC laboratories, the samples were split in equal portions, packed in polystyrene boxes containing ice-packs, to minimise degradation during transit, and then forwarded to the other participating laboratories. In the following weeks, up to seven antibiotics were measured by the three laboratories. More details are given in the section 3.3.6.

2.3. Analytical standards and chemicals

Analytical standards metronidazole, sulfamethoxazole, trimethoprim (purity > 99%) were supplied by Riedel-de Haën (Sydney, Australia). Clarithromycin (purity > 97.7%) was supplied by United States Pharmacopeia-USP, (Maryland, USA). Azythromycin and tylosin tartrate (purity > 95%) were supplied by Fluka (Sydney, Australia); clindamycin hydrochloride, roxithromicyn, (purity > 90%) were supplied by Sigma-Aldrich (Sydney, Australia). Pseudo erythromycin-A enol ether (dehydro-erythromycin, purity > 98%) and the deuterated standards [^{2}H4] sulfamethoxazole (sulfamethoxazole-d_4), [^{2}H9] trimethoprim (trimethoprim-d_9), [^{2}H3] clarithromycin (clarithromycin-d_3) and [^{2}H3] azithromycin (azithromycin-d_3) were supplied by Toronto Research Chemicals (North York, Canada). Isotope enrichment was > 98% for all the deuterated standards. Methanol (MeOH) (ChromAR HPLC grade) was purchased from Mallinckrodt (New Jersey, USA); ethyl acetate (EtAc), formic acid

(purity 99%) was purchased from Ajax FineChem (Sydney, Australia). The ultra pure water (H_2O) used for laboratory purposes as well as LC mobile phase was purified using a IBIS Technology (Perth, Australia) Ion Exchange System followed by Elga Purelab Ultra System (Sydney, Australia). Glass fiber filters (GF/F, 0.45 μm) were purchased from Whatman (Clifton, USA). Stock solutions (nominal concentration of 1 μg/μL) were prepared by dissolving a known amount of an analytical standard or a surrogate standard in MeOH/H_2O (30:70 v:v). The stock solution of clarithromycin was prepared in acetone due to its low solubility in MeOH/H_2O. Two working solutions (nominal concentration 10 ng/μL and 1 ng/μL) containing all the analytical standards were prepared freshly for each analytical run by serial dilution of the single compound stock solutions. A working solution (nominal concentration 10 ng/μL) containing all the deuterated standards was prepared bi-monthly. All the solutions, as well as analytical and surrogate standards were kept in a commercial refrigerator at 0–4 ºC to avoid degradation.

2.4. Solid-Phase Extraction pre-concentration

The solid-phase extraction (SPE) procedure was adapted from Göbel *et al.* [21]. Briefly, secondary effluent samples (250 mL) were filtered through 0.45 μm glass fiber filters and then diluted to 500 mL with ultra pure water. Post- RO water samples were already subject to microfiltration/reverse osmosis treatment in the water recycling facilities, and therefore did not require further filtration. A 500 mL aliquot was processed through the SPE cartridges. An appropriate surrogate standard mix containing the deuterated standards was also spiked before SPE extraction (typically ranging between 25–50 ng/L for post- RO water and 200 ng/L for secondary effluent) to determine recoveries and to correct for matrix effects. All SPE pre-concentration used Oasis HLB (6mL, 500 mg) cartridges (Oasis, Waters), with an automated Aspec XLi extractor (Gilson, Middleton, USA) used for the conditioning, washing, and elution steps. SPE conditions are also reported (Table 2). Samples were homogenized and then loaded onto the SPE cartridges using three 8-channel off-line peristaltic pumps (Gilson) at a flow rate of 3 mL/min. After loading and washing, the cartridges were gently dried under vacuum in a manifold system for 20–30 minutes and were then stored in a freezer (- 18 ºC). For elution, the cartridges were left to defrost at room temperature for 3–4 hours and analytes were then eluted into 12 mL glass test tubes. The final extract (ca. 12 mL) was concentrated to near dryness in a dry block

heater fitted with nitrogen blowdown (Ratek 30D, Victoria, Australia) set at 38 °C. Extracts were resuspended in 500 µL of MeOH:H_2O (30:70 v:v) and then stored in 2 mL Teflon-lined screw cap amber glass vials at 4 °C until analysis.

2.5. LC-MS/MS method

An Agilent 1100 HPLC system (Palo Alto, USA) equipped with a solvent degasser unit, a quaternary pump and a 100 well-plate autosampler was used for the chromatographic work. LC separation was achieved with a Phenomenex (Torrance, USA) Gemini C18 column (125 mm × 3mm, 3µm) at a flow rate of 150 µL/min. Other details on the LC method are given in (Table 3), available as supplementary information. The LC was coupled to a Micromass Quattro Ultima Triple Quadrupole (Manchester, UK) system fitted with an electrospray interface (ESI) operated in positive ion mode. For optimum signal, capillary and cone voltages were 2800V and 30–45V, respectively. Other MS tuning parameters are listed in (Table 4). When instrument source and optics were perfectly clean, hexapole1, aperture and hexapole2 generally required low voltages (0.0V, 0.1V and 0.1V respectively); the drop in sensitivity observed after some use (i.e. when contamination was starting to adversely affect source and hexapoles) was corrected by increasing aperture and hexapole2 between 0.4-0.8V. Nitrogen and argon were supplied by BOC Gases, Australia. Nitrogen gas (cryogenic liquid) was used as both desolvation and nebulizer gas, while high purity argon (99.997% purity) was used as collision gas for multiple reaction monitoring (MRM) experiments. MRM was preferred over SIM (single ion monitoring) or MS scan modes because of the superior advantages offered by this technique, including high sensitivity, high selectivity, and the low risk of false positives. Through tandem-mass spectrometry experiments (MS/MS), the most intense characteristic transitions were identified for each analyte and surrogate standard (Table 5). Two transitions were used for each compound, and the MRM ratio and retention time (t_R) was also monitored over the time. Compounds were generally characterised by stable MS/MS fragmentation spectra so that identification of the main MRM transitions was very simple. To increase the sensitivity of the analytical assay, the MRM transitions were also grouped in five windows based on the t_R of each analyte. The dwell time of each *m/z* value monitored was set proportionally to the

number of transitions in that window (Table 5). The maximum number of transitions that could be recorded for each time window was 8, with a dwell time of 100 ms.

Quantitation was performed using the ratio of the peak areas of the analyte and of the surrogate standard (Table 5). An external calibration curve, plotting ratio against concentration, was obtained by diluting standards in MeOH:H_2O (30:70 v:v). Concentrations in the samples were calculated by comparing the peak area ratios of the analyte and their correspondent surrogate standard in the SPE extract, to the corresponding ratios in the standard solution. Calibration curves (usually up to six points in the appropriate concentration range) were acquired at the beginning and at the end of each batch of samples.

Instrumental and/or laboratory contamination were also monitored by regular and methodical analysis of injector and procedural blanks (laboratory blanks) as well as field and trip blanks collected during sampling. About 33% of the samples processed were blanks. In particular, analyses of injector blanks revealed that there was significant carry-over between injections of standard solutions and samples. The memory effect was more pronounced for high molecular weight macrolide antibiotics (i.e. tylosin, roxythromycin, azythromycin, erythromycin-H_2O and clarythromycin). This problem was eliminated by rinsing the injector needle in the injection port with a mixture of MeOH:EtAc (50:50 v:v) for 1 minute before and after each injection.

Data processing was carried out using MassLynx NT 4.0 software, while data quantitation was performed using QuanLynx 4.0.

3. Results and discussion

3.1. Optimization of electrospray interface (ESI) and tandem-mass spectrometry (MS/MS) parameters

ESI and MS/MS tuning parameters were optimised by continuous infusion of standards solutions of antibiotics (10 ng/μL in 50:50 (v:v) MeOH:H_2O containing 0.5% formic acid; infusion flow rate of 5–10 μL/min). Only the positive ionization mode was tested because it has been previously reported in several publications (i.e. [1, 19, 21]) that ESI(+) is the preferential ionisation mode for these analytes. The intensities of the MRM transitions were optimised by varying cone voltage, which

controls the introduction of the ions into the ion block, and collision energy, which influences the formation of fragments in the collision cell. In addition, the ion energy settings in the quadrupoles were tuned to achieve best sensitivity at unit mass resolution. Cone voltage did not particularly influence the sensitivity of the analytical determination, but collision energy required specific tuning for each analyte to ensure maximum sensitivity (Table 5).

The presence of a considerable percentage of formic acid (0.4 – 1.0 %) in the mobile phase ensured that the most intense precursor ion observed was the proton adduct $[M+H]^+$. Other characteristic precursor ions such as the sodium adduct $[M+Na]^+$ or the sodium-solvent adduct ($[M+MeOH+Na]^+$ were not suitable for MS/MS fragmentation even when present in the MS scan spectra because they produce very noisy and unstable MS/MS spectra. In general, sodium adducts are very sensitive ions in SIM mode (i.e. using single quadrupoles or triple quadrupoles in SIM mode) but are not suitable to undergo MS/MS or MRM experiments. The parent ion selected for azythromicyn was the double charged $[M+2H]^+$ at m/z= 375.3, as it resulted in the most abundant species in the MS spectra. For azythromycin, Gross *et al.* [19] selected the single charged species $[M+H]^+$ at m/z= 749 using similar ESI-MS instrumentation and mobile phase constituted of acetonitrile, methanol (2:1 v:v) and a buffer consisting of ammonium acetate, acetic acid at pH = 4.7. Under the mobile phase conditions used here, (H_2O and MeOH with high percentages of formic acid) only the double charged species was present in the MS spectra for azythromycin, highlighting the importance of the mobile phase composition in the formation of single and double charged species in ESI.

The results of these experiments in terms of fragmentation patterns were generally in agreement with those previously reported in the literature (i.e. [1, 19, 21]).

3.2. Development of the chromatographic separation

Chromatographic separation of the mixture of antibiotics was initially tested using two columns. The first column was a Phenyl-C6 column from Phenomenex (250 X 2 mm, 5 μm), which showed good performance with analytical standards and simple water matrices such as spiked ultra pure water, tap water or post-RO water. However,

this column was generally not suitable for applications involving more complex matrices such as secondary effluents due to frequent peak broadening phenomena. The second column tested was a Gemini C18 (150 X 2mm, 3 μm), which showed excellent chromatographic performance and was subsequently adopted for validation and analysis work on the mixture of antibiotics.

All the compounds showed very reproducible chromatographic peaks using the Gemini C18 column. Only metronidazole showed poor retention ($t_{R\ metronidazole}$ = 9.20 min) and occasional peak broadening, especially in secondary treated wastewater extracts.

A LC-MS/MS chromatogram showing the separation and detection of the nine analytes of interest is presented in Figure 2. The sample selected was a QC sample, consisting of 250 mL of secondary effluent diluted to 500 mL with ultra pure water and spiked with 100 ng/L of antibiotics and deuterated standards before being processed through SPE.

3.3. Validation of the analytical method

Studies concerning instrumental linearity, instrumental detection limits (IDLs), peak identification criteria (t_R and MRM ratio), accuracy, precision, method limit of quantitation (MLQ), in-house reproducibility, matrix effect as well as a round-robin test were undertaken to validate the analytical procedure. The results of these studies are presented in the following sections.

3.3.1. Instrumental linearity, instrumental detection limits and peak identification criteria

Calibration curves spanning from 10 ng/mL up to 25000 ng/mL of antibiotics were injected into the LC-MS/MS to check linearity ranges. Depending on the ionisation efficiency of each compound, calibration curves showed good linearity in the range 125–18750 pg injected (except for metronidazole which showed linearity in the range 125–6250 pg injected), typically with R^2 values higher than 0.9967 (Table 6). At higher concentration ranges, the linearity of the ESI response was often lost, probably due to the limited excess of charge available on the solvent droplets [30]. An

estimation of the instrumental detection limits (IDLs) based on multiple injections (n=10) of a 0.05 ng/μL solution is also given. IDLs were in the range 1–27 pg of analyte on column. These results were consistent with previous studies [19]. The instrumental precision based on repeat consecutive injections (n=10) of a solution at 0.05 ng/μL was also tested to assess variability of peak retention time (t_R) and variability of the peak MRM ratio. MRM ratio variability (expressed as relative standard deviation, RSD) was generally less than 10% and t_R variability was generally less than 0.3% (except for metronidazole and trimethoprim, with t_R variabilities of 2% and 1%, respectively). A methodical monitoring of the MRM ratio of the analyte in the calibration curve against the MRM ratio of the analyte in the environmental sample is an important consideration to avoid reporting false positive detections. In fact, the MRM ratio of a given analyte in the calibration curve must match within 20–30% the MRM ratio in the sample extracts [31]. If this is not the case, it is likely that the analyte of interest is co-eluting with interfering species, thus altering the native MRM ratio.

3.3.2. Solid-phase extraction recoveries: accuracy and precision

Studies concerning accuracy and precision of the analytical method were undertaken by SPE experiments on blank and spiked aqueous samples. The method was validated for both secondary effluent and post-RO water. Accuracy was expressed as recovery percentage while precision was expressed as RSD. For most of the recovery experiments (Table 7), the concentrations tested in post-RO water were 10, 50, 100 ng/L (n=5 for each spiking level plus three blanks); in secondary treated water concentrations tested were 25, 50, 100 ng/L (n=3 for each spiking level plus three blanks). No substantial differences in the recovery of the analytes were observed after spiking the water samples with different antibiotic concentrations thus recoveries are presented as average values at the different concentration levels tested in this work. The average percent recoveries of these spikes were generally greater than 89% in post-RO water and greater than 93% in secondary effluent (Table 7), except for erythromycin-H_2O which showed lower recovery percentages (53±11% and 48±8% in pre-RO and post-RO aqueous samples, respectively). Other methods utilising SPE pre-concentration have reported similar recovery ranges [19, 21, 25, 26, 32, 33]. The precision, expressed as the standard deviation of the recovery experiments, was generally acceptable, varying between 6–16% in post-RO water and 7–16% in

secondary effluent. Recovery values in secondary effluent samples were corrected by subtracting the concentration of each analyte in the unspiked samples (average of n=3 blanks) from the concentration of the analyte determined in the corresponding spiked samples. No corrections were made for post-RO samples since none of the antibiotics were detected in unspiked post-RO samples.

3.3.3. Method limits of quantitation

Low level spiking samples were processed through SPE and the results were used to estimate Method Limits of Quantitation (MLQs). MLQs were calculated from the concentration equivalent to a signal-to-noise ratio (*s/n*) of ten [29, 34] by manual *s/n* calculation on unsmoothed chromatograms using peaks of known concentration. Average sample based MLQs in post-RO water were calculated from analysis of SPE extracts of samples spiked with 10 ng/L and 50 ng/L of antibiotics, and were found to be between 2.5 and 31 ng/L (Table 7). Average sample based MLQs in treated wastewater, were calculated using a range of spiked secondary effluent samples (50–100 ng/L), and were on average higher than those reported in post- RO water, ranging between 23 and 53 ng/L. Since secondary effluent samples contained considerable amounts of antibiotics, the concentration corresponding to MLQ was calculated by downscaling the *s/n* ratio of the peak at the measured concentration and assuming a linear correlation through zero[21]. MLQs reported in this work were generally in the same order of magnitude to those previously reported in literature [19, 21]. Proposed health values were calculated by Department of Health of Western Australia using the equation used to formulate the Australian Guidelines for Water Recycling (AGWR) [18]. The lowest therapeutic dose from the pharmacopeia, with a safety factor of 100 for an adult of 70 kg of body weight and assuming 2 litres of water consumption per day was used in calculating these values. Health target MLD values were set at 10% of the AGWR (Table 7). For both post-RO water and secondary treated effluent, sample based MLDs (MLDs = MLQs / 3.33, in Table 7) achieved by the SPE LC-MS/MS method presented in this work were 3 to 6 orders of magnitude lower than the health target MLD values (last column of Table 7).

These results are comparable with other studies. For example, for the antibiotics clarithromycin, erythromycin-H_2O, roxithromycin, sulfamethoxazole and trimethoprim, Webb *et al.* [35] reported several orders of magnitude difference

between daily intake calculated from drinking water sources (<40 ng/day, 2L per day) and therapeutic dose (between 150–2000 mg/day). This suggests that the health risks from ingestion of antibiotics (or other PPCPs (i.e.[14, 15, 29, 35]) *via* drinking water are minimal.

The possibility of chronic exposure of organisms to low levels of antibiotics through augmentation of natural water supplies with treated wastewater (i.e. through SAT, GWR or RBF) has led to concerns of the development of antibiotic resistance in the environment. The minimum concentration of antibiotic which will inhibit the growth of the isolated microorganism (MIC, Minimum Inhibitory Concentration) is an important factor. For example, MIC factors of single antibiotics (i.e. sulfamethoxazole, trimethoprim, erythromycin and clindamycin) for various reference bacterial strains (*S. Aureus, E. Faecalis* and *E.Coli*) often are in the range 10^1–10^3 μg/L [36]. Thus there are several orders of magnitude of difference between the observed concentrations of antibiotics in secondary effluent (as well as post- RO treated water) and the observed MIC factors. This would imply low risk of development of antibiotic resistance in those organisms. Nevertheless, more than one compound belonging to a given class of antibiotic as well as other classes of antibiotics (e.g. fluoroquinolones, dihydrofolate reductase inhibitors, tetracyclins, beta-lactams, aminoglycosides) characterised by much lower MIC factors (i.e. MICs as low as 2 μg/L have been reported for ciprofloxacin [36]) are likely to be present in the secondary effluents (i.e. [1, 19, 21, 22, 24, 26, 33, 37]). The combination of these antimicrobial agents may well result in synergistic effects, and thus the development of antibacterial resistance should not be dismissed.

3.3.4. In-house reproducibility of sampling procedure and SPE LC-MS/MS methodology

In-house reproducibility of the analytical procedure, including water sampling, SPE extraction and LC-MS/MS analysis, was tested by duplicate measurements of 24 hour composite secondary wastewater and post-RO water samples collected on the same day (21 January 2008) at the Beenyup water recycling facility. Results from these four samples are reported (Table 8). The reproducibility data from unspiked samples is limited to a few analytes in secondary effluent only. All the antibiotics tested were below detection in the two post- RO water samples, while metronidazole,

clindamycin and erythromycin-H_2O were below detection in the secondary effluent samples. To ensure reproducibility data for all analytes, six additional Quality Control (QC) samples were also analysed within the batch of samples. QC samples included three ultra pure water spiked with 25 ng/L of antibiotics and three secondary effluent samples spiked with 100 ng/L of antibiotics. The results obtained for the QC samples are also reported (Table 8). In general, the overall RSD of the sampling procedure and analytical determination were lower than 10%, very similar to the SPE precision reported from spiked matrices (Tables 7-8).

3.3.5. Matrix effect in secondary effluent samples

Matrix components present in contaminated water samples are known to be responsible for suppressing and, less frequently, for enhancing the absolute analyte response. This often results in variable detection limits and, more importantly, erroneous quantitative results. Matrix components are thought to alter the analyte response mainly by (1) influencing the viscosity and surface tension of the mobile phase droplets, thereby reducing solvent evaporation efficiency, (2) competing with the analyte to gain or lose a charge in the API source, also limiting the ejection of charged ions from the droplets, (3) neutralization in gas phase *via* deprotonation reactions with high gas-phase basicity compounds [30]. Regardless of the strategy adopted, matrix effects must be addressed to avoid loss of sensitivity, precision and accuracy, which are fundamental aspects of an analytical method. There are several different approaches proposed in the literature to correct for matrix effects. These include specific sample preparation strategies (e.g. SPE), use of surrogate standards, standard addition methods, and dilution of the SPE extracts, as well as the "echo peak" technique [19, 30, 38, 39]. Developing specific SPE methods with the intention of "*eliminating*" matrix effects is extremely difficult since the analyte and the interferences responsible for the signal suppression often have similar polarity and retention on stationary reverse phases. Although mild SPE washing steps (i.e. small percentages of polar solvents in ultra pure water) have been shown to enhance LC-MS method performance [38], matrix effects cannot be totally eliminated by this means. In fact, increasing the percentage of organic solvent in the aqueous SPE washing solution with the intent of completely washing away the interferences causing ion suppression, usually also results in eluting the analyte of interest, with an overall loss

of sensitivity. The standard addition method is another useful technique for correcting matrix effects, but requires multiple injections of the same sample [1, 19, 29, 38]. Alternatively, dilution of the SPE extract is an interesting approach explored by Gross *et al.* [19], especially when a suitable surrogate standard is not available or fails to correct for matrix effects. However, this again requires multiple dilutions and multiple injections of the same sample, ultimately affecting the sample throughput. Similarly, reducing the sample volume injected into the LC-MS system would reduce the amount of interfering species suppressing the signal [30]. Nevertheless, the latter two techniques may not always be applicable for trace analysis [19, 30, 39]. The "echo-peak" technique proposed recently [30], involves injection of a standard followed by injection of the sample, so that analytes in the standard and in the sample would elute in a similar chromatographic region and would be subject to a similar degree of signal suppression/enhancement.

To account and correct for matrix effects in this study we chose to use deuterated standards. This approach has proven very reliable [19, 30, 38] since it is likely that the analyte and a co-eluting deuterated homologue would be subject to almost identical matrix effects. First of all, ion suppression was generally observed for all the compounds in secondary effluent extracts, however, matrix effects were automatically accounted for in the case of those analytes that were quantified with their corresponding deuterated homologue (sulfamethoxazole, azythromycin, trimethoprim and clarythromycin (Table 5)). Deuterated homologues were not available for the nitroimidazole anti-infective metronidazole and for the macrolide antibiotics tylosin, erythromycin-H_2O, roxythromycin and clindamycin. Therefore, matrix effects were further investigated for these compounds. The deuterated standard chosen for metronidazole was sulfamethoxazole-d_4, while tylosin, erythromycin-H_2O, roxythromycin and clindamycin were quantified using clarithromycin-d_3. To effectively correct for matrix effects, a surrogate standard should elute in the same chromatographic region as the target analyte and should show a similar degree of ion-enhancement/ion suppression. To verify the efficiency of the chosen surrogate standards to correct for signal changes due to ion suppression, the peak area to concentration ratio of standard calibration curves in 70:30 (v:v) MeOH:H_2O were compared to those in three different wastewater samples each spiked to a different concentration. The spiking concentrations tested were 25, 50 and 100 ng/L of antibiotics. Results for metronidazole, roxithromycin and clindamycin are shown in

Figure 3, available as supplementary information. The calibration lines (equation of the line and linearity) for spiked samples showed excellent agreement with those for standards in pure solvent, showing that the surrogate standards effectively overcome matrix effects. Similar trends were observed also for tylosin and erythromycin-H_2O (data no shown). Ongoing QC controls (data not shown) using different wastewater samples (n=15 over a 12 month period) from various Perth WWTPs confirm that the deuterated standards chosen are appropriate to correct for matrix effects.

3.3.6. Inter-laboratory trial

As part of good laboratory practice and method validation for Curtin Water Quality Research Centre (CWQRC), a laboratory comparison for the measurement of seven antibiotics was organised in May - June 2007. The trial participants were CWQRC located in Perth WA, National Measurement Institute (NMI, Sydney NSW), and DVGW-Technologiezentrum Wasser (TZW, Karlsruhe, Germany). Seven antibiotics were measured by the three laboratories. Only three analytes were measurable by all participants. These were erythromycin-H_2O, roxithromycin, and sulfamethoxazole. The results of the inter-laboratory trial are presented (Table 9). NMI and TZW laboratories are randomly named Lab1 and Lab3, while CWQRC was Lab2. There was generally good agreement in the concentrations measured by all laboratories, and no significant trends or deviations were evident for most of the compounds. The only significant difference was observed for the macrolide clarithromycin, where measured concentrations are in disagreement. Unfortunately, RSDs associated with sample measurements were not available because the samples were not processed in duplicate due to costs associated with shipping and analysing the samples. It is also interesting to note that the antibiotic concentrations in the post-RO treated waters and in the groundwater, which is from a relatively pristine protected catchment, were reported to be below the detection limits by all laboratories.

3.4. Concentrations of antibiotics in pre- and post- RO water and estimation of compounds rejection by the MF/RO plant

While the presence of a number of classes of human and veterinary antibiotics has been reported in previous studies [19, 21-24, 26-28, 33, 37, 40-42] there is a

significant lack of data concerning their presence in Australian wastewater effluents. A comprehensive study on the behaviour of antibiotics during conventional wastewater treatment followed by MF/RO filtration for a water recycling facility located in Brisbane, Australia was published recently [1]. In the present study, the antibiotics sulfamethoxazole, trimethoprim, erythromycin-H_2O, roxithromycin azithromycin and clarithromycin were detected in all the secondary treated effluents (Table 9) while metronidazole and clindamycin were detected only in 40% of the samples. Data for tylosin was not available for that specific set of effluent samples. Concentration ranges of antibiotics found in secondary effluents from Perth's metropolitan WWTPs compare well with those previously reported from several other WWTPs (i.e.[1, 19, 21-24, 26-28, 33, 43, 44], see also Table 10) suggesting that dosage and antibiotic classes prescribed are often globally very similar and that classical wastewater treatment plants are not effective in achieving complete removal of such compounds from raw influent wastewater. Data for metronidazole in secondary effluent and post-RO is scarce.

For comparison, the concentrations of each antibiotic reported by the three laboratories (Table 9) have been averaged and compared with the ADWG. Results for the most commonly detected antibiotics (i.e. metronidazole, sulfamethoxazole, trimethoprim, erythromycin-H_2O, clindamycin, roxithromycin and clarithromycin) are presented in Figure 4. The concentrations of these compounds measured in secondary wastewater effluent were three to four orders of magnitude lower than the suggested guidelines for drinking water. In contrast to pre-RO water, antibiotic concentrations in post-RO samples were always below detection limits, (MLQs reported were four to five order of magnitude lower than suggested guidelines for drinking water), suggesting that RO was efficiently removing these antibiotics from secondary treated wastewater. Watkinson *et al.* [1] have reported very small residual concentrations of some antibiotics including roxithromycin (med. 10 ng/L, frequency 100%, LOD = 1 ng/L), trimethoprim (med. 5 ng/L, frequency 100%, LOD = 1 ng/L) and tylosin (med. 1 ng/L, frequency 100%, LOD = 1 ng/L) in post- MF/RO treated water from the Brisbane water recycling facility, with overall removal efficiencies from the liquid phase higher than 90%. From our data, removal efficiencies for RO can only be estimated because most compounds could not be measured in post-RO water. However, using the post-RO water MLQ as upper bound, removal efficiencies ranged between > 91–99%. These estimated results compare very well to those calculated or

predicted for other water recycling plants. For example, Drewes *et al.* [6] predicted and verified high removal (> 90%) for antibiotics such as erythromycin, sulfamethozaxole and trimethoprim through RO rejection experiments. Similarly, Snyder *et al.* [20] reported rejection efficiencies of greater than 98% with a MF/RO pilot system for the antibiotics erythromycin-H_2O, sulfamethoxazole and trimethoprim. Further research is currently being conducted to investigate trends in concentration and to study the effective rejection properties of RO membranes. Additional data from other Perth wastewater treatment plants is being acquired to investigate possible antibiotic distribution trends around the Perth metropolitan area.

4. Conclusions

A SPE LC-MS/MS method was developed for the analysis of nine antibiotics in pre- and post-RO water samples. Validation data showed good accuracy and precision and the LODs achieved easily detected concentrations of antibiotics at levels found in secondary wastewater and were suitable for studies of the efficacy of MF/RO for further removal of these compounds. Concentration ranges found in secondary effluents from Perth WWTPs compare well with data previously published in literature. None of the nine antibiotics were detected in any post-RO treated water sample analysed, suggesting RO is an effective treatment to reduce concentrations of antibiotics in secondary treated effluents. Several orders of magnitude difference was observed between MLQs in post-RO and proposed drinking water limit guidelines calculated by Western Australian Department of Health suggesting that concerns with regards to indirect exposure of antibiotics via recycled water are minimal. Estimated RO rejection was generally higher than 91%.

5. Acknowledgements

The authors would like to thank W. Giger, M. Suter, J. Hollander from EAWAG for their valuable advice on development and validation of analytical methods. J. Murby and O. Lord from NMI (Sydney and Perth) and F. Sacher and the staff at TZW (Karlsruhe) are also acknowledged for participating in the inter-laboratory trial and for advising on QA/QC protocols. J. Berry and K. Hall from Micromass and B. Greirson from Agilent are also acknowledged for their constant and valuable technical support on LC-MS instrumentation. This study was part of the

Western Australian Premier's Collaborative Research Program "Characterising Treated Wastewater for Drinking Purposes Following Reverse Osmosis Treatment". The authors would like to thank all project partners including Water Corporation of Western Australia, CSIRO Land and Water and Department of Health at UWA. Dr L. Berwick, Prof. U. Von Gunten and Dr. K. L. Linge are also acknowledged for pre-reviewing the manuscript. We also acknowledge the contribution of two anonymous reviewers to revision and improvement of the manuscript.

References

[1] A.J. Watkinson, E.J. Murby, S.D. Costanzo, *Water Res.*, **41**, 4164 (2007).
[2] J.E. Drewes, M. Reinhard, P. Fox, *Water Res.*, **37**, 3612 (2003).
[3] OCWD Orange County Water District Official Website. http://www.ocwd.com (April 2009).
[4] PUB NEWater Official Website. http://www.pub.gov.sg/NEWater (April 2009).
[5] B.E. Nancarrow, Z. Leviston, M. Po, N.B. Porter, D.I. Tucker, *Water Sci. Technol.*, **57**, 485 (2008).
[6] J.E. Drewes, D. Sedlak, S. Snyder, E. Dickenson *Development of Indicators and Surrogate for Chemical Contaminant Removal during Wastewater Treatment and Reclamation*; WateReuse Foundation: Alexandria, VA, 2008; p 146.
[7] M.M. Huber, T.A. Ternes, U. von Gunten, *Environ. Sci. Technol.*, **38**, 5177 (2004).
[8] M.C. Dodd, H.P.E. Kohler, U. Von Gunten, *Environ. Sci. Technol.*, **43**, 2498 (2009).
[9] S. Esplugas, D.M. Bila, L.G.T. Krause, M. Dezotti, *J. Hazard. Mater.*, **149**, 631 (2007).
[10] K. Ikehata, N.J. Naghashkar, M.G. Ei-Din, *Ozone-Sci. Eng.*, **28**, 353 (2006).
[11] W. Seitz, J.Q. Jiang, W. Schulz, W.H. Weber, D. Maier, M. Maier, *Chemosphere*, **70**, 1238 (2008).
[12] J.H. Choi, R.L. Valentine, *Water Res.*, **36**, 817 (2002).
[13] E. Steinle-Darling, M. Zedda, M.H. Plumlee, H.F. Ridgway, M. Reinhard, *Water Res.*, **41**, 3959 (2007).
[14] C. Rodriguez, P. Weinstein, A. Cook, B. Devine, P. Van Buynder, *Journal of Toxicology and Environmental Health, Part A*, **70**, 1654 (2007).
[15] C. Rodriguez, A. Cook, P. Van Buynder, B. Devine, P. Weinstein, *Water Sci. Technol.*, **56**, 35 (2007).
[16] ADWG, *Australian Drinking Water Guidelines 6, National Health and Medical Research Council*. Natural Resource Management Ministerial Council: 2004.
[17] WHO, *Guidelines for Drinking-water Quality: incorporating first addendum*. 3rd ed.; World Health Organisation: Geneva, 2006; Vol. 1.
[18] AGWR, Australian Guidelines for Water Recycling: Managing Health and Environmental Risks Augmentation of Drinking Water Supplies. In Council, N. R. M. M.; Council, E. P. a. H., Eds. Biotext Pty. Ltd., Canberra: 2007.
[19] M. Gros, M. Petrovic, D. Barcelo, *Talata*, **70**, 678 (2006).
[20] S.A. Snyder, S. Adham, A.M. Redding, F.S. Cannon, J. DeCarolis, J. Oppenheimer, E.C. Wert, Y. Yoon, *Desalination*, **202**, 156 (2007).
[21] A. Gobel, C.S. McArdell, M.J.F. Suter, W. Giger, *Anal. Chem.*, **76**, 4756 (2004).
[22] W. Giger, A.C. Alder, E.M. Golet, H.P.E. Kohler, C.S. McArdell, E. Molnar, H. Siegrist, M.J.F. Suter, *Chimia*, **57**, 485 (2003).
[23] C.S. McArdell, E. Molnar, M.J.F. Suter, W. Giger, *Environ. Sci. Technol.*, **37**, 5479 (2003).
[24] X.S. Miao, F. Bishay, M. Chen, C.D. Metcalfe, *Environ. Sci. Technol.*, **38**, 3533 (2004).
[25] M.J. Hilton, K.V. Thomas, *J. Chromatogr. A*, **1015**, 129 (2003).
[26] R. Hirsch, T.A. Ternes, K. Haberer, A. Mehlich, F. Ballwanz, K.L. Kratz, *J. Chromatogr. A*, **815**, 213 (1998).
[27] K.D. Brown, J. Kulis, B. Thomson, T.H. Chapman, D.B. Mawhinney, *Sci. Total Environ.*, **366**, 772 (2006).

[28] K.G. Karthikeyan, M.T. Meyer, *Sci. Total Environ.*, **361**, 196 (2006).
[29] F. Busetti, K.L. Linge, J.W. Blythe, A. Heitz, *J. Chromatogr. A*, **1213**, 200 (2008).
[30] L.L. Jessome, D.A. Volmer, *Lc Gc N. Am.*, 83 (2006).
[31] EU, *Official Journal of the European Communities*, **L221**, 8 (2002).
[32] F. Sacher, F.T. Lang, H.J. Brauch, I. Blankenhorn, *J. Chromatogr. A*, **938**, 199 (2001).
[33] R. Hirsch, T. Ternes, K. Haberer, K.L. Kratz, *Sci. Total Environ.*, **225**, 109 (1999).
[34] J.P. Foley, J.G. Dorsey, *Chromatographia*, **18**, 503 (1984).
[35] S. Webb, T. Ternes, M. Gibert, K. Olejniczak, *Toxicol. Lett.*, **142**, 157 (2003).
[36] B. Wiedemann, H. Grimm, *Antibiotics in Laboratory Medicine*. Williams and Wilkins: Baltimore, 1996.
[37] E.M. Golet, A.C. Alder, A. Hartmann, T.A. Ternes, W. Giger, *Anal. Chem.*, **73**, 3632 (2001).
[38] T. Benijts, R. Dams, W. Lambert, A. De Leenheer, *J. Chromatogr. A*, **1029**, 153 (2004).
[39] C.R. Mallet, Z.L. Lu, J.R. Mazzeo, *Rapid Commun. Mass Spectrom.*, **18**, 49 (2004).
[40] A. Gobel, A. Thomsen, C.S. McArdell, A. Joss, W. Giger, *Environ. Sci. Technol.*, **39**, 3981 (2005).
[41] E.M. Golet, A.C. Alder, W. Giger, *Environ. Sci. Technol.*, **36**, 3645 (2002).
[42] D.W. Kolpin, E.T. Furlong, M.T. Meyer, E.M. Thurman, S.D. Zaugg, L.B. Barber, H.T. Buxton, *Environ. Sci. Technol.*, **36**, 1202 (2002).
[43] M.S. Diaz-Cruz, D. Barcelo, *Trac-Trends Anal. Chem.*, **24**, 645 (2005).
[44] S. Yang, K.H. Carlson, *J. Chromatogr. A*, **1038**, 141 (2004).

7. Figure captions

Figure 1. Flow chart of Kwinana Water Reclamation Plant (KWRP) indicating the sampling points used in this work: Panel 1 (secondary effluent, pre- MF) and Panel 7 (product water, post- RO). MF= microfiltration; RO= reverse osmosis.

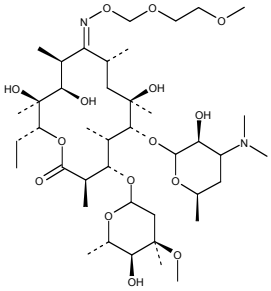
Figure 2. A typical LC-MS/MS chromatogram for separation of nine antibiotics using the Phenomenex Gemini C18 column (150 mm × 2mm, 3 μm particle size) at a flow rate of 0.15 mL/min. The sample selected was a QC sample, constituted of 250 mL of secondary effluent spiked with 100 ng/L of antibiotics before being processed through SPE.

Figure 3. Comparison of peak area ratios to concentration ratio for metronidazole (surrogate standard: sulfamethoxazole-d_4), roxithromycin and clindamycin (surrogate standard: clarithromycin-d_3), for both standard calibration curves in MeOH:H_2O 70:30 (v/v) and three spiked secondary effluent samples. Curves are almost identical, indicating that the surrogate standards chosen were able to correct ion suppression and matrix effects although they are not the exact deuterated homologues.

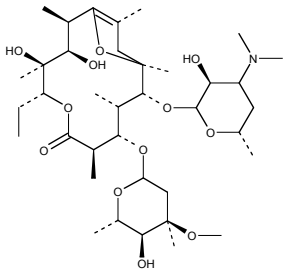
Figure 4. Concentrations of selected antibiotics in pre and post- RO samples compared to the Australian Drinking Water Guidelines calculated by Western Australian Department of Health using the equation used to formulate the draft Australian guidelines for water recycling [18]. Note that none of the antibiotics analysed were detected in post- RO water and the values reported in the figure are the actual MLQs in post- RO water.

Table 1. Name, formula, molecular weight, molecular structure, CAS number, class and application of the antibiotics investigated. H=human application; V=veterinary application

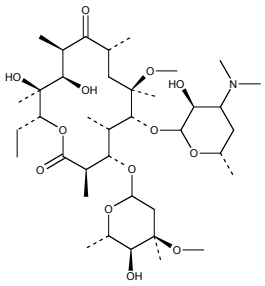
Roxithromycin
$C_{41}H_{76}N_2O_{15}$
MW: 837.10
CAS: 80214–83–1
Macrolide antibiotic
Application: H



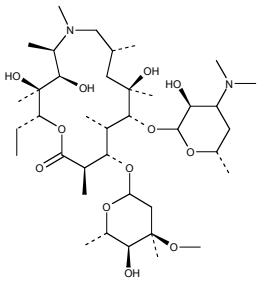
Erythromycin-H_2O
$C_{37}H_{65}NO_{12}$
MW: 715.91
CAS: 33396–29–1
Macrolide antibiotic
Application: H



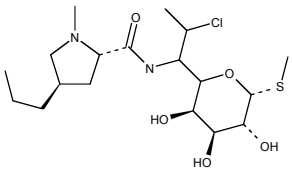
Clarithromycin
$C_{38}H_{69}NO_{13}$
MW: 747.95
CAS: 81103–11–9
Macrolide antibiotic
Application: H



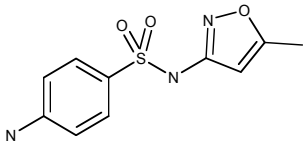
Azithromycin
$C_{38}H_{72}N_2O_{12}$
MW: 748.98
CAS: 83905–01–5
Macrolide antibiotic
Application: H



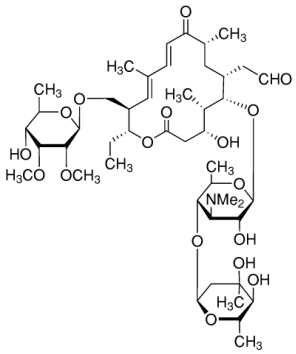
Clindamycin
$C_{18}H_{33}ClN_2O_5S$
MW: 424.98
CAS: 18323–44–9
Lincosamide antibiotic
Application: H+V



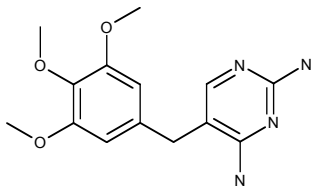
Sulfamethoxazole
$C_{10}H_{11}N_3O_3S$
MW: 253.28
CAS: 723–46–6
Sulfonamide antibiotic
Application: H



Tylosin
$C_{46}H_{77}NO_{17}$
MW: 916.53
CAS: 74610–55–2
Macrolide antibiotic
Application: H+V



Trimethoprim
$C_{14}H_{18}N_4O_3$
MW: 290.32
CAS: 738-70-5
Bacteriostatic antibiotic
Application: H+V



Metronidazole
$C_6H_9N_3O_3$
MW: 171.15
CAS: 443–48–1
Nitroimidazole anti-infective
Application: H

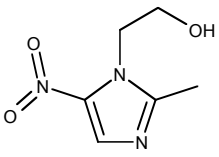


Table2. SPE conditions adopted for enrichment/clean-up of the nine antibiotics

SPE conditions	Solvents and dispensed volumes
Conditioning flow rate = 5 mL/min	6 mL MeOH:EtAc (1:1, v:v) 6 mL MeOH with 1% NH_4OH (v:v) 12 mL water (pH = 4, formic acid)
Loading flow rate = 3 mL/min	500 mL post- RO or 250 mL secondary effluent diluted to 500 mL with ultra pure water (pH = 4, formic acid)
Washing flow rate = 5 mL/min	4.5 mL water with 10% MeOH (v:v) 12 mL water (pH = 4, formic acid)
Drying/Storage	20–30 minute at 20 mmHg followed by storage at - 18 ºC
Elution* flow rate = 1 mL/min	3 mL + 3 mL MeOH with 1% formic acid (v:v) 3 mL + 3 mL MeOH:EtAc (1:1, v:v)

*Note: 3 minutes delay is applied between each aliquot dispensed. This ensures that the stationary phase is efficiently soaked with the eluting solvents.

Table 3. Liquid chromatographic parameters adopted for the separation of the nine antibiotics

Time [min]	Eluent A [%]	Eluent B [%]
0	10	90
5	10	90
10	15	85
15	40	60
20	60	40
30	70	30
32.5	100	0
55	100	0
55.1	10	90
75	10	90

LC conditions:

Eluent A: MeOH with 1% formic Acid
Eluent B: H_2O with 0.4% formic acid
Flow rate: 0.15 mL/min
Column: Gemini C18 (150mm × 2mm, 3µm)
Precolumn: Gemini C18 (4mm × 3mm, 3µm)
Injected volume: 12.5 µL of post- RO extract; 25 µL of secondary effluent extract

Table 4. General ESI(+) and MS/MS tuning parameters adopted for the detection of antibiotics

ESI-MS tuning parameters	**Setting**
Capillary voltage (V)	2800
Cone voltage (V)	30–45
Hex.1, aperture, hex.2 (V)	0.0, 0.1, 0.1
Source temperature (ºC)	130
Desolvation temperature (ºC)	325
N_2 cone gas flow (L/h)	30
N_2 desolvation gas flow (L/h)	325
Quad.1 and quad.3 resolution	1
Ion energy quad. 1, ion energy quad. 3	1.0, 1.5
Multiplier	750

Table 5. Precursor and product ions, cone voltage and collision energy values optimised for the analysis of antibiotics under ESI(+) MS/MS in MRM mode

Compound	t_R **(min)**	**Precursor ion [*m/z*]**	**Product ions [*m/z*]**	**Cone voltage**	**Dwell time (ms)**	**Collision energy**
Window 1:5.0–15.0 min						
Metronidazole[a]	9.20	172.0	82.0	30	150	25
			128.0	30	150	25
Window 2: 18.0–25.0 min						
Trimethoprim	22.34	291.3	123.3	30	150	23
			230.2	30	150	25
Trimethoprim-d_9	22.17	300.3	123.3	30	150	23
			234.2	30	150	25
Window 3: 25.1–28.0 min						
Sulfamethoxazole	26.13	254.2	108.0	45	100	23
			156.0	45	100	17
Sulfamethoxazole-d_4	26.06	258.3	112.3	45	100	23
			160.2	45	100	17
Azythromycin	25.62	375.6	434.3	45	100	20
			591.5	45	100	15
Azythromycin-d_3	25.61	376.7	437.3	45	100	20
			594.5	45	100	15
Window 4: 27.0–30.5 min						
Clindamycin[b]	27.45	425.3	126.3	45	150	25
			377.1	45	150	20
Tylosin[b]	29.14	916.5	174.1	45	150	40
			772.5	45	150	30
Window 5: 30.0–34.0 min						
Erythromycin-H_2O[b]	30.98	716.6	158.1	45	100	25
			558.5	45	100	20
Clarithromycin	31.14	748.5	158.1	45	100	30
			590.5	45	100	22
Clarithromycin-d_3	31.13	751.4	161.3	45	100	25
			593.4	45	100	20
Roxithromycin[b]	31.36	837.5	158.3	45	100	37
			679.4	45	100	25

[a] surrogate standard used for quantitation was sulfamethoxazole-d_4

[b] surrogate standard used for quantitation was clarithromycin-d_3

The product ions underlined denote the transitions used for quantification.

Table 6. Linear ranges and regression values typically observed for calibration curves; instrumental detection limits (IDLs) estimated at *s/n* = 3, instrumental precision in terms of MRM ratio and t_R (average ± RSD) were obtained from repeated injections (n=10) of a solution at 0.05 ng/μL of antibiotics

Compound	**Standard solutions**				
	Linear range tested (pg on column)	R^2	IDL *s/n*=3 (pg on column)	MRM ratio (±RSD%)	t_R (±RSD%)
Metronidazole	125–6250	0.9982	15	3.2±8	9.2±2.0
Trimethoprim	125–18750	0.9989	6	5.8±10	22.3±1.0
Sulfamethoxazole	125–18750	0.9982	7.5	1.4±10	26.1±0.3
Azythromycin	125–18750	0.9997	15	17±6	25.6±0.3
Clindamycin	125–18750	0.9995	1	2.4±6	27.4±0.2
Tylosin	125–18750	0.9967	3.5	1.4±9	29.1±0.1
Erythromycin-H_2O	125–18750	0.9997	27	2.2±5	31.0±0.2
Clarythromycin	125–18750	0.9996	2	2.1±6	31.1±0.2
Roxythromycin	125–18750	0.9997	3.5	4.3 ±8	31.4±0.2

Table 7. Accuracy, precision and MLQs in post- RO and secondary effluent achieved with the SPE LC-MS/MS method; target MLD values developed for the project are also reported

Compound	Post-RO water			Secondary effluent			Target MLD values from Health Guidelines (ng/L)
	% Accuracy (n=15)	Precision (RSD, n=15)	MLQ (ng/L): range and (average)	% Accuracy (n=9)	Precision (RSD, n=9)	MLQ (ng/L): range and (average)	
Metronidazole	114	16	15–45 (31)	112	15	25–67 (53)	350000
Trimethoprim	106	6	5–8 (6.5)	112	11	25–90 (52)	70000
Sulfamethoxazole	94	6	5–17 (9)	101	12	25–100 (53)	35000
Azythromycin	100 [a]	13 [a]	10–21 (15)	106 [aa]	16 [aa]	25–85 (50)	4000
Clindamycin	112	11	5–12 (8)	93	9	15–40 (23)	300000
Tylosin	102 [b]	13 [b]	5–8.5 (7.5)	100 [bb]	7 [bb]	18–50 (27)	1050000
Erythromycin-H_2O	48 [c]	8 [c]	7–20 (10)	53 [cc]	11 [cc]	35–50 (45)	35000
Clarithromycin	97	7	3–20 (7.5)	97	8	10–60 (30)	250000
Roxithromycin	89	12	1–3.5 (2.5)	95	7	5–85 (32)	150000

[a] n=12, spiked concentration between 10–50 ng/L
[aa] n=9, spiked concentration between 100–250 ng/L
[b] n=6, spiked concentration between 50–100 ng/L
[bb] n=6, spiked concentration 50 ng/L
[c] n=6, spiked concentration between 10–50 ng/L
[cc] n=9, spiked concentration 100 ng/L

Table 8. Reproducibility of the SPE LC-MS/MS method on real samples (i.e. treated wastewater and post-RO water) sampled on the 21 January 2008 from Beenyup water treatment plant. QC samples are also included and were a triplicate of secondary effluent spiked at 100 ng/L and a triplicate of ultra pure water spiked at 25 ng/L

Compound	Beenyup secondary effluent		Beenyup post- RO	
	Average (ng/L) ±RSD	QC samples: Recovery (%) ±RSD	Average (ng/L) ±RSD	QC samples: Recovery (%) ±RSD
Metronidazole	< MLQ	77±10	< MLQ	130±3
Trimethoprim	216±9	92±3	< MLQ	102±7
Sulfamethoxazole	304±2	92±2	< MLQ	99±3
Azythromycin	206±4	125±5	< MLQ	106±6
Clindamycin	< MLQ	80±2	< MLQ	74±4
Tylosin	21±7	105±6	< MLQ	103± 6
Erythromycin-H_2O	< MLQ	63±1	< MLQ	55±3
Clarithromycin	142±2	105±2	< MLQ	95±5
Roxithromycin	150±7	92±3	< MLQ	103±8

Table 9. Inter-laboratory comparison for the analysis of selected antibiotics: aqueous samples for the trial were one groundwater sample, five secondary treated wastewater samples, and three post- RO treated water samples collected from various locations around the city of Perth

Aqueous samples	Metronidazole			Sulfamethoxazole			Trimethoprim			Clindamycin			Erythromycin-H_2O			Roxithromycin			Clarithromycin		
	Lab 1	Lab 2	Lab 3	Lab 1	Lab 2	Lab 3	Lab 1	Lab 2	Lab 3	Lab 1	Lab 2	Lab 3	Lab 1	Lab 2	Lab 3	Lab 1	Lab 2	Lab 3	Lab 1	Lab 2	Lab 3
W1_Grab_240507 [a]	< 50	< 25	...	< 50	< 50	< 2	< 25	< 25	...	< 50	< 45	...	< 50	< 50	< 25	< 50	< 50	< 50	< 50	< 50	...
K_P6_Grab_040607 [b]	< 50	< 25	...	680	580	390	290	325	...	< 50	< 45	...	290	360	340	220	375	370	110	270	...
K_P7_Grab_040607 [c]	< 20	< 15	...	< 20	< 5	< 2	< 10	< 5	...	< 20	< 5	...	< 20	< 20	< 25	< 20	< 1	< 50	< 20	< 20	...
K_P6_Grab_070607 [b]	< 50	< 25	...	620	670	380	310	400	...	< 50	< 45	...	330	400	340	240	350	340	110	225	...
K_P7_Grab_070607 [c]	< 10	< 15	...	< 10	< 5	< 2	< 5	< 5	...	< 10	< 5	...	< 10	< 20	< 25	< 10	< 1	< 50	< 10	< 20	...
B_WW_Comp_120607 [b]	51	61	...	390	400	320	140	170	...	62	52	...	1000	930	750	240	300	290	170	280	...
S_WW_Comp_190607 [b]	74	71	...	540	540	440	420	485	...	65	49	...	1100	930	920	340	345	380	200	290	...
K_P6_Comp_300507 [b]	...	< 25	...	...	490	410	...	454	...	...	< 45	...	...	330	320	...	341	310	...	210	...
K_P7_Comp_300507 [c]	...	< 15	...	...	< 5	< 2	...	< 5	...	...	< 5	...	...	< 20	< 25	...	< 1	< 50	...	< 20	...

[a] Groundwater

[b] Secondary treated effluent

[c] Post- RO treated water

Table 10. Concentration of antibiotics in secondary effluent and post- RO samples from different locations around the world. RO rejection is also reported when possible

Compound	Secondary effluent (ng/L)	Post Reverse Osmosis (ng/L)	Estimated RO rejection (%)	Country	References
Metronidazole	< 25–71	< 15	n.a.	Australia	This study
Trimethoprim	170[a]	…	…	USA	[28]
	180	…	…	USA	[27]
	320[b]	…	…	Germany	[33]
	70–310	…	…	Spain	[19]
	86–170	…	…	Switzerland	[21]
	468	2.1	99.5	USA	[6]
	80	5	93.7	Australia	[1]
	186	< 1	> 99.5	USA	[20]
	170–485	< 5	> 98.9	Australia	This study
Sulfamethoxazole	200[a]	…	…	USA	[28]
	310	…	…	USA	[27]
	400[b]	…	…	Germany	[33]
	< 20–820	…	…	Spain	[19]
	243[c]	…	…	Canada	[24]
	344–352	…	…	Switzerland	[21]
	939	2.0	99.8	USA	[6]
	255	< 3	> 98.8	Australia	[1]
	90	1.2	98.7	USA	[20]
	400–680	< 5	> 99.1	Australia	This study
Azithromycin	110–129	…	…	Switzerland	[21]
	50–210	…	…	Spain	[19]
	< 50–160	< 10	n.a.	Australia	This study
Clindamycin	1	< 1	91	Australia	[1]
	< 45–65	< 5	n.a.	Australia	This study
Roxithromycin	< 5–31	…	…	Switzerland	[23]
	680[b]	…	…	Germany	[33]
	11–21	…	…	Switzerland	[21]
	8[c]	…	…	Canada	[24]
	…	…	> 90	USA	[6]
	140	10	92.8	Australia	[1]
	300–390	< 1	> 99.7	Australia	This study
Erythromycin-H_2O	…	…	> 90	USA	[6]
	270[a]	…	…	USA	[28]
	2500[b]	…	…	Germany	[33]
	80[c]	…	…	Canada	[24]
	< 20–199	…	…	Switzerland	[23]
	<6	…	…	Spain	[19]
	54–96	…	…	Switzerland	[21]
	detected	detected	n.a.	Australia	[1]
	336	< 1	> 99.7	USA	[20]
	360–930	< 20	> 94.5	Australia	This study
Clarithromycin	188–374	…	…	Switzerland	[21]
	87[c]	…	…	Canada	[24]
	57–328	…	…	Switzerland	[23]
	210–290	< 20	> 91.4	Australia	This study
Tylosin	60	…	…	USA	[44]
	20	1	95	Australia	[1]

[a] n=10 WWTP effluent samples
[b] n=10 WWTP effluent samples
[c] n=8 WWTP effluent samples

Figure 1.

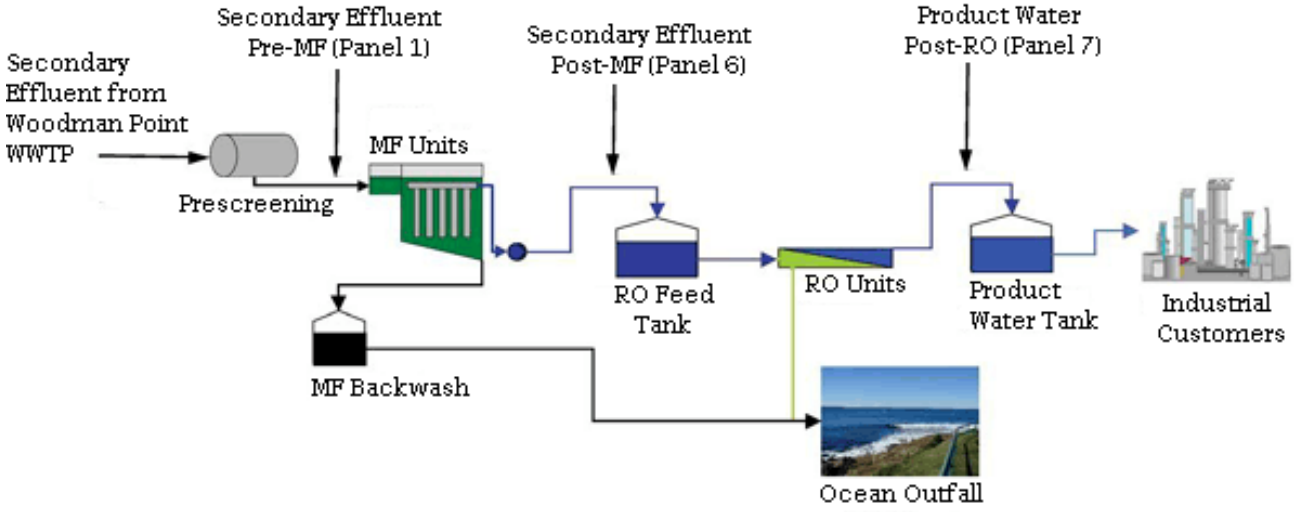

Secondary Effluent from Woodman Point WWTP
Secondary Effluent Pre-MF (Panel 1)
Prescreening
MF Units
MF Backwash
Secondary Effluent Post-MF (Panel 6)
RO Feed Tank
RO Units
Product Water Post-RO (Panel 7)
Product Water Tank
Industrial Customers
Ocean Outfall

Figure 2.

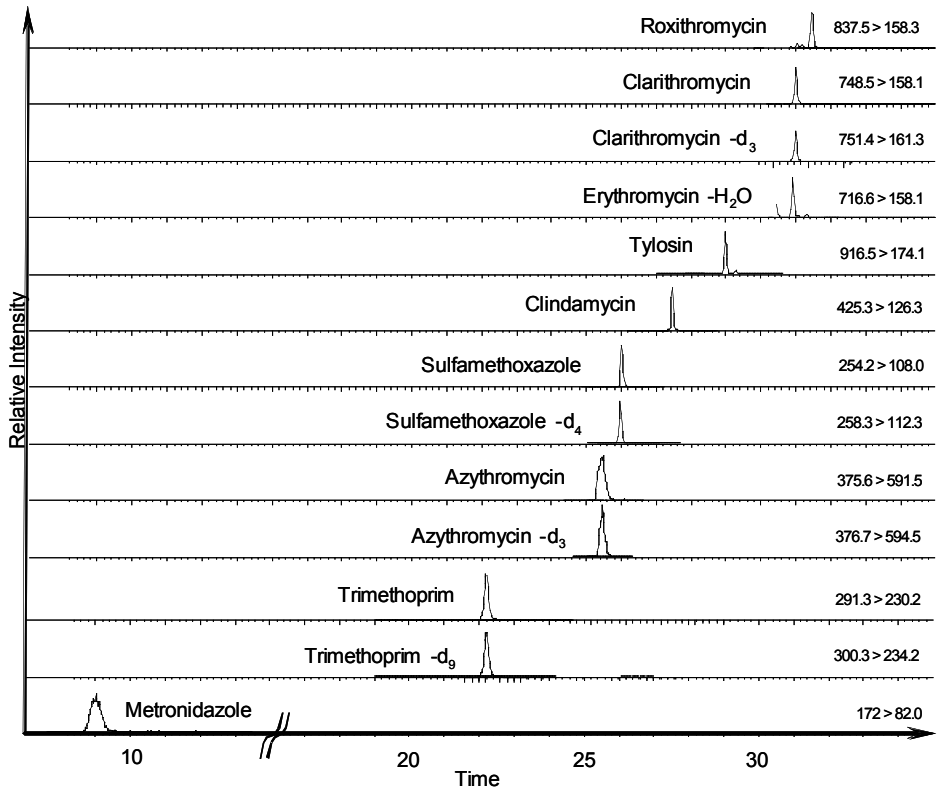
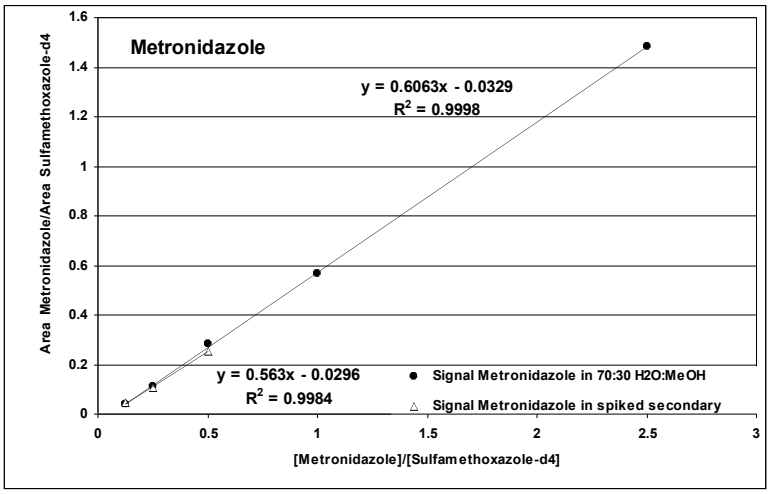
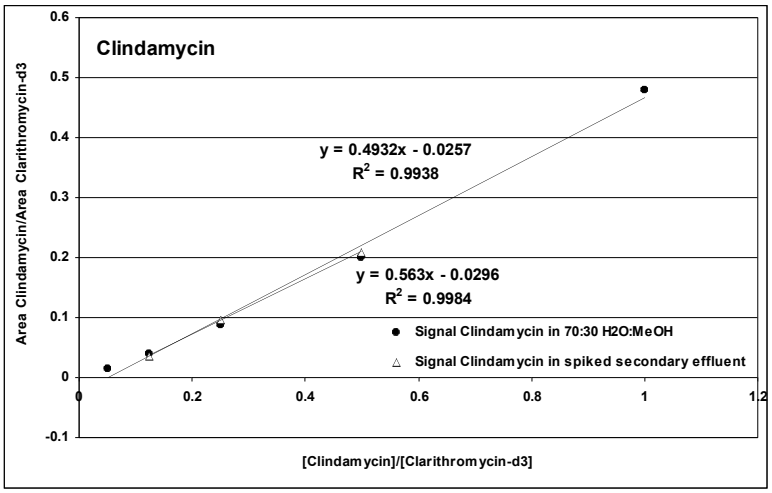

Roxithromycin
837.5 > 158.3
Clarithromycin
748.5 > 158.1
Clarithromycin -d3
751.4 > 161.3
Erythromycin -H2O
716.6 > 158.1
Tylosin
916.5 > 174.1
Clindamycin
425.3 > 126.3
Sulfamethoxazole
254.2 > 108.0
Sulfamethoxazole -d4
258.3 > 112.3
Azythromycin
375.6 > 591.5
Azythromycin -d3
376.7 > 594.5
Trimethoprim
291.3 > 230.2
Trimethoprim -d9
300.3 > 234.2
Metronidazole
172 > 82.0
Relative Intensity
10
20
25
30
Time

Figure 3.


Metronidazole
y = 0.6063x - 0.0329
R2 = 0.9998
y = 0.563x - 0.0296
R2 = 0.9984
Signal Metronidazole in 70:30 H2O:MeOH
Signal Metronidazole in spiked secondary
Area Metronidazole/Area Sulfamethoxazole-d4
[Metronidazole]/[Sulfamethoxazole-d4]


Clindamycin
y = 0.4932x - 0.0257
R2 = 0.9938
y = 0.563x - 0.0296
R2 = 0.9984
Signal Clindamycin in 70:30 H2O:MeOH
Signal Clindamycin in spiked secondary effluent
Area Clindamycin/Area Clarithromycin-d3
[Clindamycin]/[Clarithromycin-d3]

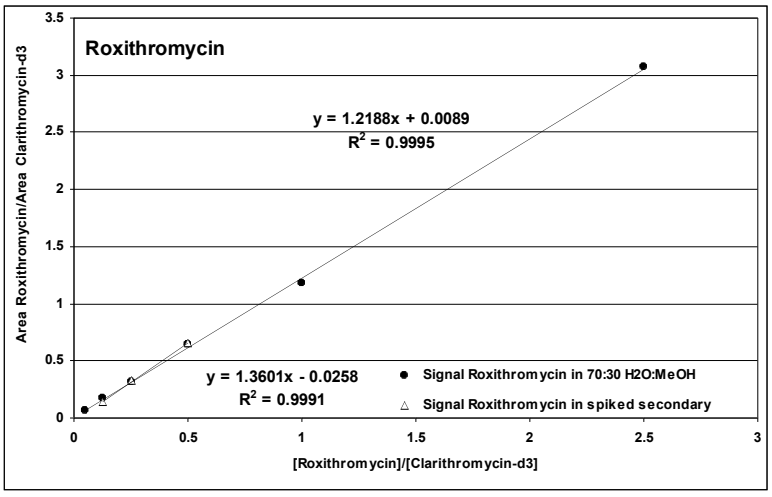
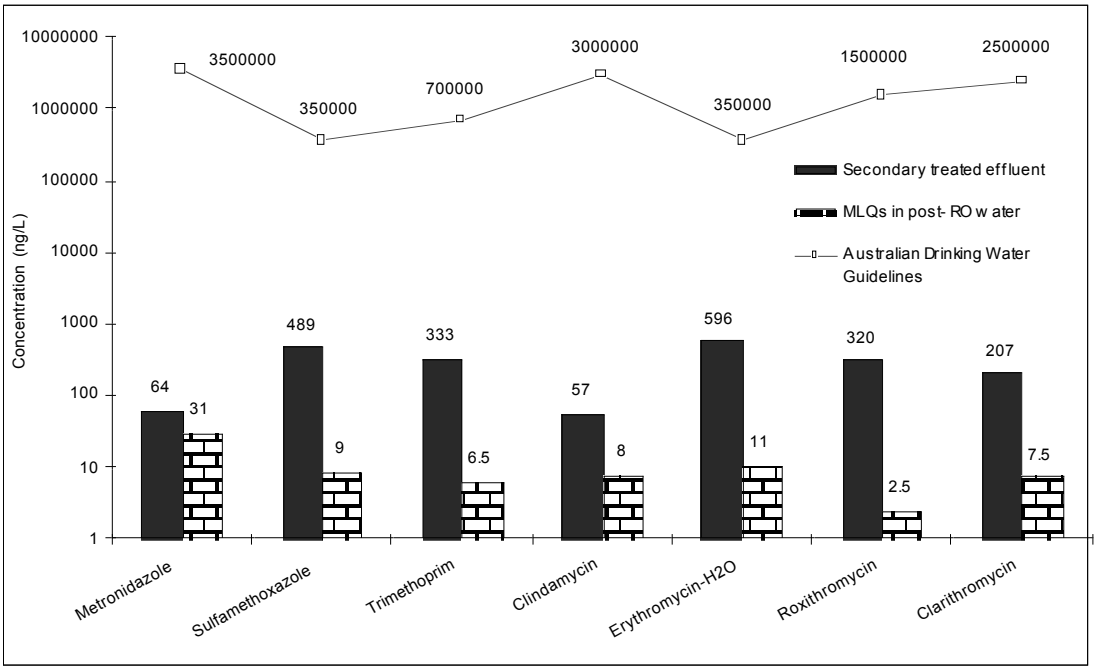

Roxithromycin
y = 1.2188x + 0.0089
R2 = 0.9995
y = 1.3601x - 0.0258
R2 = 0.9991
Signal Roxithromycin in 70:30 H2O:MeOH
Signal Roxithromycin in spiked secondary
Area Roxithromycin/Area Clarithromycin-d3
[Roxithromycin]/[Clarithromycin-d3]

Figure 4.


10000000
1000000
100000
10000
1000
100
10
1
Concentration (ng/L)
3500000
350000
700000
3000000
350000
1500000
2500000
Secondary treated effluent
MLQs in post- RO water
Australian Drinking Water Guidelines
64
31
489
9
333
6.5
57
8
596
11
320
2.5
207
7.5
Metronidazole
Sulfamethoxazole
Trimethoprim
Clindamycin
Erythromycin-H2O
Roxithromycin
Clarithromycin