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Pharmaceutical pollution in marine waters and benthic flora of the southern Australian coastline

Benjamin M. Long[A,*], Samantha Harriage[A], Nick L. Schultz[A], Craig D. H. Sherman[B] and Michael Thomas[C]

Environmental context. Most human pharmaceutical waste is discharged to the environment. While the presence of pharmaceuticals in freshwater systems is well documented globally, little is known of the impact on marine ecosystems. We measured pharmaceuticals in a marine environment in south-eastern Australia and found pharmaceutical concentrations around 24 000 times higher in benthic flora than in the marine surface waters. We discuss the potential use of seaweeds as biological indicators of pharmaceutical pollution.

For full list of author affiliations and declarations see end of paper

*Correspondence to:
Benjamin M. Long
Future Regions Research Centre, Federation University Australia, Mt Helen, Vic. 3350, Australia
Email: bm.long@federation.edu.au

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ABSTRACT

Rationale. Pharmaceuticals are emerging pollutants of concern with a range of adverse consequences for organisms and ecosystems. Their presence in freshwater and estuarine systems has been well documented, but less is known about their prevalence in open ocean, or their uptake by benthic flora. This preliminary survey of the southern Australian coastline sought to measure the concentrations of key pharmaceuticals in both surface waters and benthic flora. **Methodology.** This study used LC-MS/MS to measure the concentration carbamazepine, tramadol and venlafaxine in (1) samples from wastewater treatment plants, (2) ocean surface waters and (3) several species of benthic flora. Surface waters and benthic flora were sampled at two sites near waste water treatment plant (WWTP) discharges, and one site away from any discharge. **Results.** All three pharmaceuticals were detected in surface water samples with their risk assessed (via risk quotient) as medium risk (carbamazepine) or low risk (venlafaxine, tramadol). All three pharmaceuticals were also detected in benthic flora, particularly in brown macroalgae; Tramadol was measured at a maximum of 34.7 ng g^{-1} in *Hormosira banksii*, and Venlafaxine was recorded at a maximum of 17.3 ng g^{-1} in *Caulocystis cephalornithos*. **Discussion.** The calculated bioconcentration factors suggest the pharmaceutical concentrations in benthic flora were up to ~24 000 times higher than in surrounding surface water. There was also evidence that proximity to WWTP outfalls influenced the levels of pharmaceuticals in benthic flora. The results suggest that the benthic flora may be suitable bioindicators of pharmaceutical contamination and that the potential impacts of pharmaceutical pollutants in marine ecosystems demand further investigation.

Keywords: benthic flora, bioindicators, emerging contaminant, macroalgae, marine, pharmaceutical pollution, risk assessment, risk quotient.

Introduction

Pharmaceuticals are often classed as emerging contaminants of concern (Madikizela *et al.* 2020; Branchet *et al.* 2021), though they have been documented since the 1970s (Hignite and Azarnoff 1977) and throughout the late 20th century (Halling-Sørensen *et al.* 1998). The observed effects of pharmaceutical contaminants in aquatic systems include additive acute and chronic toxicity (mostly in fish) with a range of physiological consequences (see Mostofa *et al.* 2013 for a review), and ecosystem-level effects can include reduced biodiversity at different trophic levels. Human usage of pharmaceuticals is the key source of these contaminants, and wastewater treatment plants (WWTPs) are considered the primary vector to the environment (Madikizela *et al.* 2020). The sewerage system and

wastewater treatment plants are not designed to remove trace organic contaminants and thus they are passed to the environment via discharge points (e.g. ocean outfalls) (Meyer *et al.* 2019).

The presence of pharmaceuticals in river systems is increasingly well characterised worldwide (Katsikaros and Chrysikopoulos 2021; Adeleye *et al.* 2022) and in Australia (Scott *et al.* 2014; Roberts *et al.* 2016; South Australian Environmental Protection Agency SA-EPA 2019). Pharmaceutical concentrations have been characterised in marine surface water and sediments (Fabbri and Franzellitti 2016; Madikizela *et al.* 2020; Branchet *et al.* 2021), though the presence and fate of pharmaceuticals in the marine environment is less well known. Discharge into the open ocean is a common way to eliminate treated wastewater where large dilution volumes aid in reducing potentially hazardous concentrations of contaminants. A recent review by Madikizela *et al.* (2020) summarised the results of marine and estuary pharmaceutical monitoring of 42 studies, with up to 151 pharmaceuticals detected in the ng L^{-1} to μg L^{-1} range worldwide. In Australia, there have been reports of pharmaceutical monitoring in large estuaries, including Darling Harbour, Sydney (Birch *et al.* 2015; Anim *et al.* 2020), Brisbane Harbour (Anim *et al.* 2020), the Yarra River, Melbourne (Anim *et al.* 2020) and Darwin Harbour (French *et al.* 2015). To date there have been no published studies of pharmaceuticals in open ocean surrounding Australia despite Australia having 140 monitored ocean outfalls (Rohmana *et al.* 2021).

Pharmaceuticals have been quantified in commercially farmed bivalves, including mussels (Álvarez-Muñoz *et al.* 2015; Świacka *et al.* 2019) and clams (Maranho *et al.* 2015), and in marine macroalgae such as *Saccharina latissima* and *Laminaria digitata* (Álvarez-Muñoz *et al.* 2015), and *Enteromorpha* sp., *Turbinaria conoides* and *Ulva lactuca* (Ali *et al.* 2018). The mechanisms of pharmaceutical uptake by marine macroalgae are poorly understood, as highlighted by Ali *et al.* (2018), with few data to facilitate our understanding.

This study sought to undertake a preliminary survey in the southern Australian ocean for concentrations of three commonly detected pharmaceuticals that have low removal from wastewater and are persistent in natural ecosystems (carbamazepine, tramadol and venlafaxine). These three neuroactive pharmaceuticals have been frequently detected in Australian baseline studies and hence were chosen as suitable markers of pharmaceutical contamination (Scott *et al.* 2014; Birch *et al.* 2015; French *et al.* 2015; Roberts *et al.* 2016; South Australian Environmental Protection Agency SA-EPA 2019; Anim *et al.* 2020). Carbamazepine is a psychiatric drug with minimal biotic and abiotic degradation in natural ecosystems. As such, it is a recalcitrant and ubiquitous pollutant to which many aquatic ecosystems are exposed. Exposure can alter behaviour, immune response, growth, fecundity, and community structure (Quinn *et al.* 2008; Lamichhane *et al.* 2013; Jarvis *et al.* 2014). Tramadol is a synthetic opioid that can influence animal behaviour even at low concentrations (Santos *et al.* 2021). Venlafaxine is an antidepressant that can influence embryonic development in fish (Painter *et al.* 2009) and is potentially toxic to higher plants (Feito *et al.* 2013). Carbamazepine, venlafaxine and tramadol are an issue globally as they are consistently found above or close to minimum response concentrations and are hence likely to have adverse effects on flora and fauna (Duarte *et al.* 2022; Wilkinson *et al.* 2022).

This survey was conducted through direct measurement of pharmaceuticals in surface waters and benthic flora. It was envisioned that as benthic flora are in constant contact with potentially contaminated water they could act as effective bioindicators. To the author's knowledge this study is the first in Australia to survey pharmaceutical pollution in the open ocean and the first to use marine benthic flora as a bioindicator for pharmaceutical pollution.

Experimental

Sample sites

Three sample sites were chosen along the southern Australian coastline in the state of Victoria (Fig. 1). The coastline is within the Bass Strait region and is typically affected by swell, currents and winds from the Southern Ocean.

Sampling occurred on the 9th of March 2021 where the locations have an average temperature of 16.8°C, average rainfall of 37.4 mm and average wind speed of 16.5 km h^{-1} (tending from SSW) based on Australian Bureau of Meteorology weather statistics collected at an Airey's Inlet weather station (−38.46°, 144.09°). For the month of March the average significant wave height is 1.50 m with a period of 7.1 s and a swell direction of 182° (S) based on data collected by the Apollo Bay Wave Buoy (−38.75°, 143.72°). Wave data was provided by the Victorian Coastal Monitoring Program with funding through Department of Environment, Land, Water and Planning, University of Melbourne and Deakin University.

The Breamlea sample site (Fig. 1) was chosen for its proximity to a large WWTP with an ocean outfall. The treatment plant services the City of Greater Geelong (Fig. 1) and its surrounding area. The WWTP services approximately 270 000 people (median age 40) (Australian Bureau of Statistics 2016) and discharges 51.6 ML day^{-1} only at programmed times (T. Murphy pers. comm. 2021) treating a population equivalent of 350 000 (W. McCance pers. comm. 2022). The discharge point is an underwater diffuser 800–1500 m offshore. The beach is a recreational surf beach.

The Lorne sample site (Fig. 1) was chosen for its proximity to a small WWTP and an economically important coastal town for the tourism industry. The WWTP services approximately 1000 people (Median age 54) (Australian Bureau of Statistics 2016) and discharges 0.7 ML day^{-1} only at

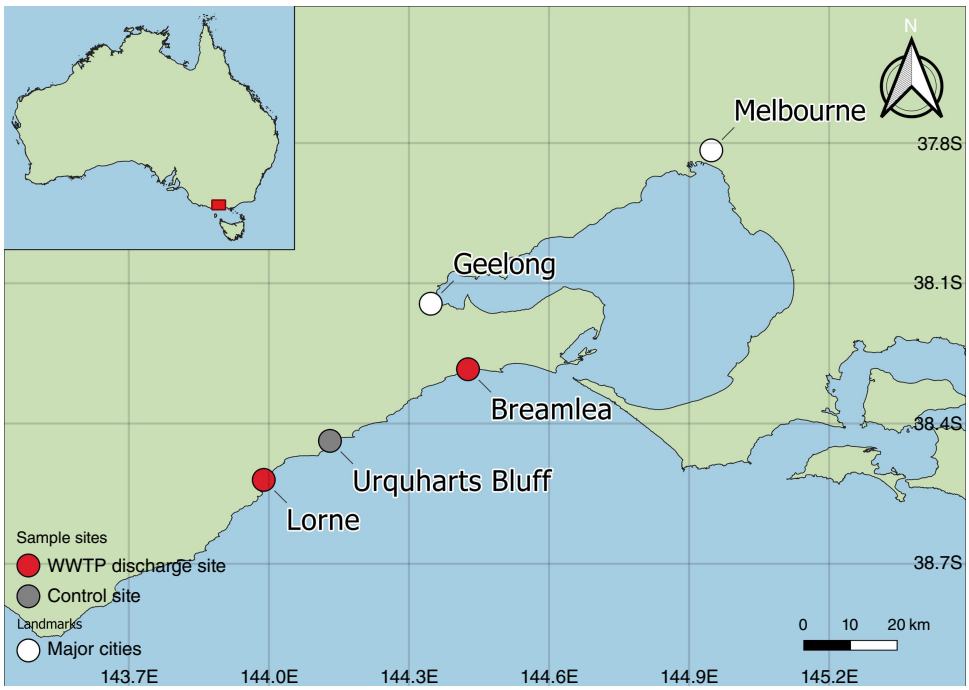

Fig. 1. Map of study locations in southern Victoria, Australia (Inset: Australia). Sampling sites near known WWTP discharge indicated with red circles and sampling sites with no known WWTP impact indicated with grey circles. Major cities indicated with white circles. Map developed using QGIS software and Australian Bureau of Statistics Digital Boundaries Files (Australian Bureau of Statistics 2021).

programmed times (T. Murphy pers. comm. 2021) treating a population equivalent of 3100 (W. McCance pers. comm. 2022). The discharge point is directly onto a sandy beach and to the ocean via a manmade cleavage in the rock shelf.

Urquhart's Bluff sample site was chosen as a control site as it is in a region with no known WWTP discharges. The nearest WWTP with discharge (Anglesea WWTP) is 7 km to the northeast or 6 km to the west in an overflow event (Airey's Inlet WWTP). The site is a recreational surf beach with facilities for boating and fishing. The beach has toilet amenities with a septic tank not connected to water authority sewer infrastructure.

Sample collection

Samples were collected in March 2021 during a low tidal event (~0.5 m). Triplicate water samples were collected as 500 mL grab samples in polypropylene containers from rockpools open to the ocean water low in the intertidal zone (i.e. submerged under high tide conditions). We sampled benthic flora from the same rockpools, based on local availability, including attached macroalgae and seagrass. Five species of benthic flora were sampled across the three sites, including three species of brown algae (*Hormosira banksii*, *Caulocystis cephalornithos* and *Cystophora subfarcinata*), one seaweed (*Codium* sp.) and one seagrass (*Amphibolis antarctica*), though not all species were present at each site. Both surface water and flora samples were held at 0°C before storing at −20°C. WWTP water samples were collected by ALS Limited as grab samples of final effluent.

Sample preparation – surface water

Water samples were defrosted from −20 to 4°C and pH-adjusted to a pH between 3 and 4. Phenomenex Strata octadecylsilane (C18) cartridges were then pre-conditioned with 10 mL of methanol before the addition of 1 mL of 4 mM hydrochloric acid. A 100 mL aliquot was taken from the samples and drawn through the cartridges at a flow of less than 2 mL min^{-1}. Once processed, 2.5 mL of methanol, followed by 2.5 mL of acetonitrile was passed through the cartridges to elute the sample into a 6 mL test tube before being placed under a flow of nitrogen gas to evaporate the solvent. The residue was reconstituted in a 1 mL solution containing 3% acetonitrile/water and 10 ng mL^{-1} deuterated isotope standards of carbamazepine-d_{10}, tramadol-d_3-^{13}C and venlafaxine-d_6. The solutions were filtered using 0.45 μm filters directly into HPLC vials for analysis.

Sample preparation – benthic flora

Flora samples were defrosted and dried at 40°C, 0.2 g of the dried, ground flora material was weighed out and 10 mL of methanol was introduced. The sample was vortexed for

1 min, then sonicated for 10 min. The sample was then centrifuged for 10 min at 5000 rpm and the supernatant decanted. The process was repeated two more times with acetonitrile, and the supernatants were combined. The subsequent 30 mL of methanol/acetonitrile was placed under a flow of nitrogen gas at 40°C until evaporated. The residue was then dissolved in 20 mL of a 10 ng mL^{-1} deuterated isotope solution containing carbamazepine-d_{10}, tramadol-d_3-^{13}C and venlafaxine-d_6. A 1 mL aliquot of the solutions was filtered using 0.45 µm nylon filters directly into HPLC vials for analysis.

Analysis and LC/MS parameters

The analysis of the surface water and flora species was based on the method by Birch *et al.* (2015). The analysis was conducted using a Shimazdu LCMS-8030 with a kinetex C18 column. The eluent strength gradient was increased from 3% acetonitrile in H_2O with 0.1% formic acid to 100% acetonitrile with 0.1% formic acid over 11 min. The run was then held at 100% acetonitrile with 0.1% formic acid for a further 4 min. The column was re-equilibrated for 5 min before the next injection. The needle wash solution was a 1/1 ratio of methanol/MilliQ water followed by pure MilliQ water. In terms of the mass spectrometer (MS), the nebulising gas flow was at a rate of 2 L min^{-1}, the heating block temperature was 400°C, the DL temperature was at 250°C and the drying gas flow was at 15 L min^{-1}.

Multiple reaction mode (MRM) optimisation

The MRM was optimised for the following pharmaceuticals using 100 ng mL^{-1} standards: tramadol, tramadol-d_3-^{13}C, carbamazepine, carbamazepine-d_{10}, venlafaxine and venlafaxine-d_6. One ion was used for quantification and a second ion used to confirm the identity of the compound.

Pharmaceutical standards and quantification

The pharmaceutical standards were prepared from ampule sealed samples purchased from Novachem™. The samples were dissolved in ethanol or methanol depending on solubility. Mixed pharmaceutical standards were prepared to cover a range of 0.05–50 ng mL^{-1}. Deuterated isotopes of carbamazepine, tramadol and venlafaxine were included in both the standards and the samples in an isotope dilution protocol to reduce matrix effects and to characterise those pharmaceuticals with greater accuracy (Du *et al.* 2012). Calibration curves were constructed using linear regression with r values of >0.999.

QA/QC

Throughout the analysis, a MilliQ water instrument blank and field blanks were analysed to ensure there was no carry over and standards were re-run to ensure precision and accuracy. The limit of quantification (LOQ) for pharmaceuticals was 0.5 ng L^{-1} in surface water and 5 ng g^{-1} in benthic flora. The limit of detection (LOD) was evaluated as a signal to noise ratio >3. Method recoveries measured for carbamazepine and venlafaxine were found to be 103% (±6%) and 56% (±8%), respectively. Birch *et al.* has shown that tramadol has a 54% recovery using similar methodology (Birch *et al.* 2015).

Statistical analysis

Shapiro–Wilk tests were used to check for the normal distribution of pharmaceutical concentrations from the WWTPs, surface waters and the benthic flora samples. The data from the WWTP were normally distributed and *t*-tests were used to test for differences in pharmaceutical concentrations between the two WWTPs. The data for surface waters and benthic flora were not normally distributed and so non-parametric tests were applied. To test the difference in the levels of pharmaceuticals of surface water among the three sites, Kruskal–Wallis tests were used with a *post hoc* Dunn test and a Bonferroni *P*-value adjustment for pairwise comparisons. Similarly, Dunn tests were used to test the difference in pharmaceutical concentrations in benthic flora for any species for which a pharmaceutical was recorded at multiple sites. All tests were performed using R (R Core Team 2021) and the packages dplyr (Wickham *et al.* 2020) and FSA (Ogle *et al.* 2021). Statistical analysis included values extrapolated below the LOQ for comparison of means. Samples where the detected concentration was below the LOD were included as zeros. Reported concentration ranges reflect absolute values measured.

Risk quotients

Risk quotients (RQ) were calculated using the protocol outlined by Zhou *et al.* (2019) (Eqn 1), however the concentrations involved in this study do not allow for an optimised risk quotient.

$$\mathrm{RQ} = \frac{\mathrm{MEC}}{\mathrm{PNEC}} \quad (1)$$

The measured environmental concentrations (MEC) were as calculated in this study and the predicted no effect concentrations (PNEC) were used as described by Zhou *et al.* (2019). Zhou *et al.* (2019) defined the PNEC values through a literature screening approach using chronic no-observed effect concentrations for crustaceans (carbamazepine) and fish (venlafaxine), or EC_{50} values for algae (tramadol). The PNEC values included in this study were: carbamazepine (PNEC = 10 ng L^{-1}), tramadol (PNEC = 959 ng L^{-1}) and venlafaxine (PNEC = 91.9 ng L^{-1}). Thresholds for high risk (RQ > 1), moderate risk (1 < RQ < 0.1) and low risk (RQ < 0.1) were defined as per a recent study by Gani *et al.* (2021).

Results

WWTP discharge

Pharmaceutical concentrations in the WWTP discharge are presented in Table 1. The highest recorded concentration was carbamazepine at Breamlea WWTP (860 ng L^{-1}) and the lowest was carbamazepine at Lorne WWTP (440 ng L^{-1}). The concentration of carbamazepine was significantly higher at Breamlea WWTP than at Lorne ($P < 0.001$).

Surface water

Across all three sample sites there was a total of 15 detections of pharmaceuticals from nine samples (Table 2). Carbamazepine was detected in three samples across two sites. The highest concentration of carbamazepine (0.99 ng L^{-1}) was detected at Urquhart's Bluff and there were no detections at Lorne. Tramadol was detected in seven samples across all sites. The highest concentration of tramadol (3.68 ng L^{-1}) was noted at the Urquhart's Bluff site and lowest measurable concentration (0.9 ng L^{-1}) at the Breamlea site. Venlafaxine was detected in six samples across all sites. The highest concentration of venlafaxine (1.44 ng L^{-1}) was at the Breamlea site and lowest concentration (0.52 ng L^{-1}) at the Urquhart's Bluff site. There were no significant differences in pharmaceutical concentrations among sites for any of the three pharmaceuticals ($P > 0.05$ for all pairwise comparisons).

Based on the maximum surface water concentrations measured, risk quotients were calculated according to the method by Zhou *et al.* (2019). Carbamazepine was classified as a moderate risk (RQ = 0.1) and both tramadol (RQ = 0.006) and venlafaxine (RQ = 0.02) were classified as low risk.

Benthic flora

There were 24 detections of pharmaceuticals in the benthic flora from 27 samples (Table 3). There were 16 detections of tramadol, 10 detections of venlafaxine, and 5 detections of carbamazepine. However, no detections of carbamazepine were above the limit of quantification. Tramadol was detected at all three sites. The highest detected concentration of tramadol (34.67 ng g^{-1}) was in *Hormosira banksii* at the Breamlea site. The lowest quantifiable measurement (6.21 ng g^{-1}) was in *Hormosira banksii* at the Lorne site. The concentration of tramadol in *Hormosira banksii* was significantly greater at Breamlea than at Lorne ($P = 0.026$). Venlafaxine was quantifiable at both the Breamlea and Lorne sites but was not detected at Urquhart's Bluff. The highest detected concentration of venlafaxine (17.3 ng g^{-1}) was in a *Caulocystis cephalornithos* at the Breamlea site while the lowest quantifiable measurement (8.2 ng g^{-1}) was in *Hormosira banksii* at the Urquhart's Bluff site.

Discussion

The data presented in this study demonstrate that emerging pharmaceutical pollutants are detectible in ocean surface waters and benthic flora near the source of WWTP effluent. While a direct link from the source to the water and flora sampled cannot be confirmed, the sites closest to the WWTP outfalls demonstrated higher concentrations of pharmaceuticals in macroalgae than the sites situated away from large WWTP outfalls. The data suggest that these are emerging pollutants of concern for the southern Australian benthic environment that require monitoring and further research into the potential ecological impacts of these pollutants.

Table 1. WWTP water concentrations of pharmaceuticals (ng L^{-1}) (standard errors in parentheses).

Pharmaceutical	Breamlea WWTP			Lorne WWTP			*P* value (comparison of means)
	Range	Mean	Frequency	Range	Mean	Frequency	
Carbamazepine	807–893	860 (27)	3/3	425–454	440 (8.2)	3/3	<0.001
Tramadol	359–525	455 (50)	3/3	414–703	514 (94)	3/3	0.61
Venlafaxine	490–598	560 (35)	3/3	441–559	489 (36)	3/3	0.23

Table 2. Surface water concentrations of pharmaceuticals (ng L^{-1}) including range, mean (standard error) and frequency.

Pharmaceutical	Breamlea			Lorne			Urquhart's Bluff		
	Range	Mean	Frequency	Range	Mean	Frequency	Range	Mean	Frequency
Carbamazepine	< LOQ–0.50	0.3 (0.11)	3/3	< LOQ	0.2 (0.06)	3/3	< LOQ–0.99	0.53 (0.24)	3/3
Tramadol	0.90–2.05	1.46 (0.33)	3/3	n.d.–1.76	0.9 (0.51)	2/3	n.d.–3.68	2.07 (1.09)	2/3
Venlafaxine	0.89–1.44	1.16 (0.16)	3/3	n.d.–0.78	0.26 (0.26)	1/3	n.d.–0.93	0.49 (0.27)	2/3

Where frequency is the number of samples analysed with pharmaceutical content above the limit of detection.
< LOQ, below the limit of quantification; n.d., not detected.

Table 3. Pharmaceutical concentrations (ng g^{-1} dry weight) in benthic flora including range, mean (standard error) and frequency.

Species	Breamlea			Lorne			Urquhart's Bluff		
Pharmaceutical	**Range**	**Mean**	**Frequency**	**Range**	**Mean**	**Frequency**	**Range**	**Mean**	**Frequency**
Hormosira banksii									
Carbamazepine	n.d.–< LOQ	1.03 (1.03)	1/3	n.d.	n.d.	0/6	n.d.	n.d.	0/3
Tramadol	14.38–34.67	25.94 (6.02)	3/3	n.d.–10.43	5.05 (1.71)	4/6	6.32–8.64	7.35 (0.68)	3/3
Venlafaxine	8.26–16.46	13.42 (2.59)	3/3	n.d.–< LOQ	2.32 (0.8)	4/6	n.d.	n.d.	0/3
Caulocystis cephalornithos									
Carbamazepine	n.d.–< LOQ	0.44 (0.44)	1/3	–	–	–	–	–	–
Tramadol	n.d.–16.88	5.63 (5.63)	1/3	–	–	–	–	–	–
Venlafaxine	n.d.–17.30	10.53 (5.33)	2/3	–	–	–	–	–	–
Cystosphora subfarcinata									
Carbamazepine	–	–	–	< LOQ	n/a	1/1	n.d.–< LOQ	0.39 (0.39)	1/3
Tramadol	–	–	–	18.14	n/a	1/1	< LOQ–7.99	6.38 (1.05)	3/3
Venlafaxine	–	–	–	< LOQ	n/a	1/1	n.d.	n.d.	0/3
Codium sp.									
Carbamazepine	n.d.–< LOQ	1 (1)	1/3	–	–	–	–	–	–
Tramadol	n.d.	n.d.	0/3	–	–	–	–	–	–
Venlafaxine	n.d.	n.d.	0/3	–	–	–	–	–	–
Amphibolis antarctica									
Carbamazepine	–	–	–	–	–	–	n.d.	n.d.	0/2
Tramadol	–	–	–	–	–	–	n.d.–< LOQ	2.42 (2.42)	1/2
Venlafaxine	–	–	–	–	–	–	n.d.	n.d.	0/2

Where frequency is the number of samples analysed with pharmaceutical content above the limit of detection.
< LOQ, below the limit of quantification; n.d., not detected.

WWTP discharge

The Breamlea WWTP discharges 51.6 ML day^{-1} of treated wastewater (containing 860 ng L^{-1} carbamazepine, 455 ng L^{-1} tramadol and 560 ng L^{-1} venlafaxine) and the Lorne WWTP discharges 0.7 ML day^{-1} (containing 440 ng L^{-1} carbamazepine, 514 ng L^{-1} tramadol and 489 ng L^{-1} venlafaxine). The concentrations of tramadol and venlafaxine are similar at both sites however there was a significantly lower carbamazepine concentration at the Lorne WWTP. This may be due to population demographics, WWTP design or simply an artifact of grab sampling. Carbamazepine levels in WWTP effluent have been recorded at lower levels than presented here (studies in China (Ben *et al.* 2018) and Colombia (Botero-Coy *et al.* 2018) found maximum concentrations of 55 and 78 ng L^{-1}, respectively), though the levels reported here are within the range reported from a study in Spain (Jelic *et al.* 2011) of 400–1400 ng L^{-1} in which the WWTP was designed to treat equivalent population sizes.

Surface water

Despite significant dilution in the marine environment, the focal pharmaceuticals are still detectable in surface water through standard grab sampling techniques. The maximum measured value for carbamazepine was measured at 0.99 ng L^{-1} at Urquhart's Bluff and the lowest detected concentration (0.09 ng L^{-1}) at Lorne. In Australian estuaries, carbamazepine ranges from 2.7 ng L^{-1} in Sydney estuary (Birch *et al.* 2015) to as high as 550 ng L^{-1} in Darwin Harbour (French *et al.* 2015). Globally, carbamazepine in the open ocean has been previously characterised (Madikizela *et al.* 2020), with recent detections off the coasts of Tunisia (max. 0.5 ng L^{-1}; Afsa *et al.* 2020), Spain (max. 0.1 ng L^{-1}; Biel-Maeso *et al.* 2018) and France (max. 0.140 ng L^{-1}; Martínez Bueno *et al.* 2016). The concentrations of carbamazepine presented in this study are generally lower than the ranges reported in Australian estuary studies but are similar to the ranges reported in global oceanic studies.

Tramadol was measured at a maximum concentration of 5.72 ng L^{-1} at Urquhart's Bluff, but was not detected in some samples at both Lorne and Urquhart's Bluff. Darwin Harbour and Brisbane estuaries noted high concentrations of tramadol (270 and 81.9 ng L^{-1} respectively; French *et al.* 2015; Anim *et al.* 2020), while the Yarra river estuary (8.9 ng L^{-1}; Anim *et al.* 2020) and Sydney estuary (1.3 ng L^{-1}; Birch *et al.* 2015; Anim *et al.* 2020)

concentrations are similar to concentrations reported in this study, despite there being no known wastewater inputs directly to Sydney estuary (Birch *et al.* 2015). Tramadol has not been well reported globally, with Alygizakis *et al.* (2016) reporting a maximum concentration of 1 ng L^{-1} in the Saronic Gulf, Greece and Wahlberg *et al.* (2011) reporting an average value of 14 ng L^{-1} surrounding Stockholm, Sweden.

The range of venlafaxine detected was 0.31–1.6 ng L^{-1}. These values were low in comparison to all Australian estuary detections; 260 ng L^{-1} in Darwin Harbour (French *et al.* 2015), 86.2 ng L^{-1} in Brisbane Estuary (Anim *et al.* 2020), 10.0 ng L^{-1} in Yarra River Estuary (Anim *et al.* 2020) and 5.2 ng L^{-1} in Sydney Estuary (Birch *et al.* 2015; Anim *et al.* 2020). Globally, venlafaxine has been poorly represented but has been detected as high as 291 ng L^{-1} in coastal waters in North-Western Spain (Fernández-Rubio *et al.* 2019). There was a noted high variability between the three triplicates in all water samples collected, and in many cases for venlafaxine and tramadol the concentrations were below the limit of detection for our methodology. The grab sampling technique employed may account for the high variability, as samples were collected from individual rockpools with potential for microenvironments to form (i.e. concentration through evaporation or degradation due to higher temperature). However, these effects were likely minimised as samples were collected on a cool day and from a location with low exposure time above the intertidal mark. This variability highlights the need for suitable bioindicators that reflect longer-term pharmaceutical concentrations in marine environments.

Urquhart's bluff site included high individual measurements for all three pharmaceuticals. This site was chosen as a control site as there was no wastewater discharge within 7 km. However, it is possible that these pharmaceuticals are present due to a prevailing southerly swell which would carry any discharge from Lorne. There is also a septic tank system at the site which could leech into the nearby water. Further testing of sites away from outfalls is needed to determine how proximity to outfalls influences pharmaceutical concentrations in marine surface waters.

At each site, the risk quotient for each pharmaceutical in surface waters was less than one for the maximum noted concentrations. The risk quotients suggest a moderate risk of harm from carbamazepine exposure and low risk of harm from tramadol and venlafaxine exposure to marine organisms based on PNEC values. Nevertheless, as emerging contaminants, more research is needed on the potential ecological impacts of long-term low-level exposure to such pharmaceuticals (Khan *et al.* 2021), their mixtures (Srain *et al.* 2021) and the role that these low-level concentrations might have at transferring pharmaceuticals into food webs (Richmond *et al.* 2018).

Benthic flora

The potential of benthic flora as bioindicators for environmental contaminants has been recognised globally (Álvarez-Muñoz *et al.* 2015; Maranho *et al.* 2015; Ali *et al.* 2018; Świacka *et al.* 2019), though few suitable candidates from the southern Australian coastline have been identified. While globally distributed species such as *Ulva lactuca* grow in southern Australian waters (Womersley 1984) and were studied by Ali *et al.* (2018) it was not found at any of our field sites.

Our only detection of carbamazepine in benthic flora was below the limit of quantification. This is consistent with previous studies; Ali *et al.* (2018) detected carbamazepine at low levels (max. 1.7 ng g^{-1} dry weight) in macroalgae in the Saudi Red Sea, and Álvarez-Muñoz *et al.* (2015) did not detect carbamazepine in macroalgae in European coastal waters. In both those studies, carbamazepine was detected in bivalves and fish. Álvarez-Muñoz *et al.* (2015) also did not detect venlafaxine in their samples, again despite its detection in fish and bivalves. Conversely, our results suggest tramadol is readily uptaken by the macroalgae species studied. To the authors knowledge tramadol uptake by macroalgae has not been reported elsewhere.

The maximum concentration of any pharmaceutical found in the benthic flora was tramadol in *Hormosira Banksii* at Breamlea (34.7 ng g^{-1}). Using a bioconcentration factor (BCF) model outlined by Arnot and Gobas (2006), this equates to a BCF of ~24 000 L kg^{-1} and hence a 24 000 times increase in concentration. This accumulation was also noted in other species where pharmaceuticals were detected, most notably venlafaxine and tramadol in *Caulocystis* sp. at Breamlea. These high concentrations also indicate that these algae bioaccumulate tramadol and the other pharmaceuticals by the European Union definition of bioaccumulation (European Commision 2006). The benthic flora accumulate the pharmaceuticals to higher levels than found in the surface water, and while the RQ calculated for the surface water indicate that there is likely to be no negative effects on marine organisms, this may not be true for marine species who feed on benthic flora.

Hormosira banksii has previously been suggested as a bioindicator of wastewater influence as the species is sensitive to wastewater components (e.g. ammonia), such that its recruitment and growth is inhibited in the presence of wastewater (Doblin and Clayton 1995). Our data suggest that this species is a suitable indicator of pharmaceutical presence, and it would be useful to test the sensitivity of the species to a broad range of pharmaceutical concentrations.

Hormosira Banksii at the Breamlea site showed significantly higher concentrations of tramadol than the Lorne site, and this may reflect the higher concentration of pharmaceuticals in the surface waters over a long timescale and be related to higher WWTP discharge volumes at Breamlea. This strengthens the assumption that WWTP discharge

impacts the benthic environment that is close in proximity to discharge sites. Nevertheless, the detection of pharmaceuticals in both surface water and flora at Urquhart's Bluff, away from known discharge sites, suggests that the zone of influence of pharmaceuticals requires further investigation.

The benthic flora results also highlight the potential variability in pharmaceutical uptake, both among different pharmaceuticals and among macroalgae species. A such, the analysis of a broader range of macroalgae species is warranted, both in the search for suitable bioindicators, and to observe the potential ecological impacts of pharmaceuticals in marine environments.

Conclusion

The survey suggested through both surface water and benthic flora samples that all sites were contaminated with pharmaceutical pollution. The measurement of pharmaceutical concentration in benthic flora suggested that the Breamlea site near a large Victorian WWTP has higher levels of pharmaceutical pollution than other sites along the coastline on a longer timescale. As such, our study highlights that benthic flora such as *Hormosira banksii* can be utilised as bioindicator tools for the marine environment. Benthic flora are often perennial (Schiel and Taylor 1999), they are well adapted to survive in the rough seas and constantly changing conditions of the southern Victorian coastline, and we have shown they readily uptake pharmaceuticals.

The potential impacts of pharmaceuticals in the marine environment warrant future investigations, including the combined effects of pharmaceutical pollution and other environmental stressors, such as ocean acidification (Freitas *et al.* 2016) and ocean temperatures. It is the intent of the authors to expand upon this initial investigation to investigate seasonality and fluctuating ocean conditions though passive/composite sampling.

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Data availability. Final measured values, detector responses, recoveries, calibration curves and R markdown files are available via figshare. DOI: 10.25955/20506704.

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Author affiliations

[A]Future Regions Research Centre, Federation University Australia, Mt Helen, Vic. 3350, Australia.

[B]School of Life and Environmental Sciences, Deakin University, Waurn Ponds, Vic. 3219, Australia.

[C]Research and Development, Barwon Water, Geelong, Vic. 3220, Australia.