

Sewage-derived nitrogen dispersal and N-fixation in Port Phillip Bay in south-eastern Australia

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ABSTRACT

Context. Extreme values of nitrogen isotope ratios ($\delta^{15}\text{N}$) in biota identify areas influenced by sewage discharges and nitrogen fixation. **Aims.** To measure the ecological footprint of the Western (sewage) Treatment Plant (WTP) by using $\delta^{15}\text{N}$ measurements and an NPZ model, and to identify regions where nitrogen is fixed in Port Phillip Bay, in south-eastern Australia. **Methods.** $\delta^{15}\text{N}$ was measured *in situ* for four intertidal species at 27 sites around the perimeter of the bay and simultaneously in an alga and a mussel deployed at 42 sites throughout the bay. The intertidal species were also transplanted from an area of low ^{15}N to areas of high ^{15}N . **Key results.** $\delta^{15}\text{N}$ was elevated for all species adjacent to the WTP and along the north-western shoreline of Port Phillip Bay and for ~10–15 km offshore. Transplanted algae adopted the $\delta^{15}\text{N}$ of their new environment in 6–12 weeks, whereas the half-life of tissue turnover of ^{15}N in intertidal filter-feeders was 90–234 days. Nitrogen fixation contributed to N available in two geographically isolated seagrass-dominated bays. **Conclusions.** Spatial patterns of $\delta^{15}\text{N}$ of deployed alga and phytoplankton-consuming mussels were similar to the modelled distribution of dissolved inorganic nitrogen and the modelled distribution of flagellates, respectively. **Implications.** Preferred species for on-going N monitoring are identified.

Keywords: algae, $\delta^{15}\text{N}$, eutrophication, invertebrates, modelling, nutrient dynamics, N-fixation, seagrass, stable isotopes.

Introduction

Dissolved inorganic nitrogen (DIN) is the limiting nutrient in most marine systems (Ryther and Dunstan 1971), including Port Phillip Bay (Harris *et al.* 1996). DIN is required to sustain marine productivity, but excessive concentrations have detrimental environmental effects. The positive effects of nutrients on the productivity of Port Phillip Bay were evident when input of DIN was reduced by a prolonged drought (1997–2010) that coincided with upgraded sewage treatment at the Western Treatment Plant (WTP). This sustained reduction in DIN inputs resulted in a 69% reduction in fish biomass in central Port Phillip Bay (Parry and Hirst 2016) and in an 80–90% reduction in seagrass cover at locations where DIN became limiting (Ball *et al.* 2014; Hirst *et al.* 2016). Detrimental effects of high nutrients in Port Phillip Bay include establishment of exotic algae that require higher nutrient conditions than do native species (Carnell and Keough 2014) and noxious algal blooms (Parry *et al.* 1989). Elevated nutrient concentrations near the WTP also cause increases of attached and drifting seaweed, with a high ^{15}N signal (Rodriguez 2019), at least 10 km offshore on the north-west of the bay (Chidgley and Edmunds 1997; Rodriguez 2019). In the 1980s, large amounts of this seaweed washed ashore on beaches on the north-west of Port Phillip Bay (Davies and Brown 1986), but this high marine productivity did not lead to low dissolved oxygen near the seabed (Mickelson 1990), even near WTP outlets, where infaunal diversity and biomass remained high (Poore and Kudenov 1978; Coleman and Gason 2000). Sewage treatment upgrades resulted in a ~90% decline in the amount of drifting seaweed near the WTP between 1995 and 2018 (Rodriguez 2019).

Understanding the main sources of DIN, their dispersal and temporal variation is key to understanding the ecology of many marine systems. Nutrient footprints may be determined

by direct measurements of nutrient concentrations, distributions of algae and fauna, computer models or with nitrogen isotopes. The latter requires distinctive isotope signals, but all methods have weaknesses such as cost, model quality or seasonal influences. For example, during the Port Phillip Bay study in 1969–1973 (Melbourne and Metropolitan Board of Works 1973), direct measurements showed that nutrient concentrations were elevated near WTP, and benthic fauna within ~10 km offshore was distinct from that elsewhere, but the distribution of this fauna also correlated well with sediment type and depth (Poore and Kudenov 1978; Currie and Parry 1999). In this paper, the nutrient footprint of the WTP is estimated using elevated $\delta^{15}\text{N}$ in biota, supported by nutrient measurements and a calibrated NPZ model.

Nitrogen isotope tracking ($^{15}\text{N}/^{14}\text{N}$ or $\delta^{15}\text{N}$), which integrates exposure to nitrogen over weeks to months, has become widely used around sources with a distinctive ^{15}N signature (Fong 2008; Connolly *et al.* 2009). Low values of $\delta^{15}\text{N}$ result from nitrogen fixation (N-fixation) (Welsh 2000), whereas high values result from isotopic fractionation during secondary and tertiary sewage treatment (Costanzo *et al.* 2001; Savage 2005; Thornber *et al.* 2008). Fractionation occurs because $^{14}\text{NH}_3$ volatilises more readily than does $^{15}\text{NH}_3$, and $^{14}\text{NO}_3^-$ is reduced more readily than is $^{15}\text{NO}_3^-$ by denitrifying bacteria (McClelland and Valiela 1998; deBruyn and Rasmussen 2002; Savage 2005). High $\delta^{15}\text{N}$ values in biota near sewage outfalls provide a time-integrated tracer of the plume (Costanzo *et al.* 2001; Gartner *et al.* 2002; Thornber *et al.* 2008) and the transfer of nutrients into the food web (Hansson *et al.* 1997; Gaston *et al.* 2004; Pitt *et al.* 2009; Miller *et al.* 2010). Sedentary taxa, including macroalgae (Costanzo *et al.* 2001, 2005; Gartner *et al.* 2002; Rogers 2003; Savage and Elmgren 2004; Fertig *et al.* 2009; Morris *et al.* 2009; Dailer *et al.* 2010, 2012; Parry 2021) and filter-feeders, including ascidians and sponges (Gartner *et al.* 2002), and bivalves (Rogers 2003; Carmichael *et al.* 2004; Piola *et al.* 2006; Gustafson *et al.* 2007; Fertig *et al.* 2009) have all been used to measure the biological footprint of sewage-derived ^{15}N .

In this study, $\delta^{15}\text{N}$ was measured *in situ* by using four intertidal species around the perimeter of the bay and an alga and mussels deployed throughout the bay. The four intertidal species were also transplanted from a site with a low $\delta^{15}\text{N}$ to sites near the WTP where $\delta^{15}\text{N}$ was expected to be high. The period the alga and mussels were deployed was modelled using the *Bubbles* NPZ model (Black *et al.* 2016, 2022; Jenkins and Black 2019, 2021).

The objectives of the study were as follows:

1. To use multiple techniques to establish the footprint of nutrients from a sewerage-treatment plant (WTP) on Port Phillip Bay, including identification of sites with a low $\delta^{15}\text{N}$ where nitrogen fixation may be important.
2. To estimate the time periods over which ^{15}N is integrated by different species.

3. To investigate species suitable for ongoing cost-efficient monitoring of the nutrient footprint of WTP.

Materials and methods

Study site

Port Phillip Bay is a 1930 km² marine embayment in south-eastern Australia. The Yarra River, which passes through Melbourne, enters the north of the bay, whereas the bay is connected to Bass Strait by a narrow (3 km) entrance to the south (Fig. 1). The Western Treatment Plant (WTP) treats sewage from 55% of Melbourne's population of 5 million and discharges ~400 ML day⁻¹ of tertiary sewage at four outlets located along 10 km of the north-western shoreline of Port Phillip Bay in the Geelong Arm (Fig. 1). The WTP is the world's largest lagoon sewage treatment plant (108 km²); however, between 2004 and 2012, approximately half the sewage was treated by activated sludge plants (ASPs). Most effluent is discharged from the northernmost outlet (15 East), which receives all the effluent from the ASPs and some effluent from the lagoons. The other three outlets (Lake Borrie, 145 West and Murtcaim) received effluent only from the extensive lagoon system, and the residence time of sewage in these lagoons is longer (up to 50 days).

The outfalls discharge the treated effluent close to shore onto inter-tidal sand flats. The freshwater discharge is buoyant and thereby highly influenced by wind. The circulation in the bay is three-dimensional with up/downwelling around the shorelines (Black *et al.* 1993), but the WTP plume often travels along the north-western shoreline towards the Yarra River, especially during the winter when the winds are mostly from the westerly quadrant. Similar behaviour is seen in this paper because the experiments were conducted in winter from early July to early September (Table 1).

Nutrient, freshwater and $\delta^{15}\text{N}$ inputs and bay nutrients

Between July and September 2012, while *Botryocladia* and *Mytilus* were deployed in Port Phillip Bay, salinity, nutrient concentrations (NO_x , NH_4^+ , PO_4^{3-}) and $\delta^{15}\text{N}$ of NO_3^- and NH_4^+ were measured at nine major discharges (Table 1a), whereas salinity and nutrients were measured at 10 intertidal sites on the western shoreline and at the 42 offshore mooring sites (Fig. 1, Table 1b). The frequency of each of these measurements is shown in Table 1a, b. Flow rates of all discharges (measured by Melbourne Water, and at Altona STP by City West Water) and mean DIN concentrations were used to calculate the mean daily load of ammonium, nitrate and of $\delta^{15}\text{N}$ (Table 2).

Salinity was measured in the laboratory with a YSI Pro2030, calibrated against 0.582% KCl solution. Nutrient concentrations, and $\delta^{15}\text{N}$ of NO_3^- and NH_4^+ , were measured using water samples filtered in the field by using 0.45 μm

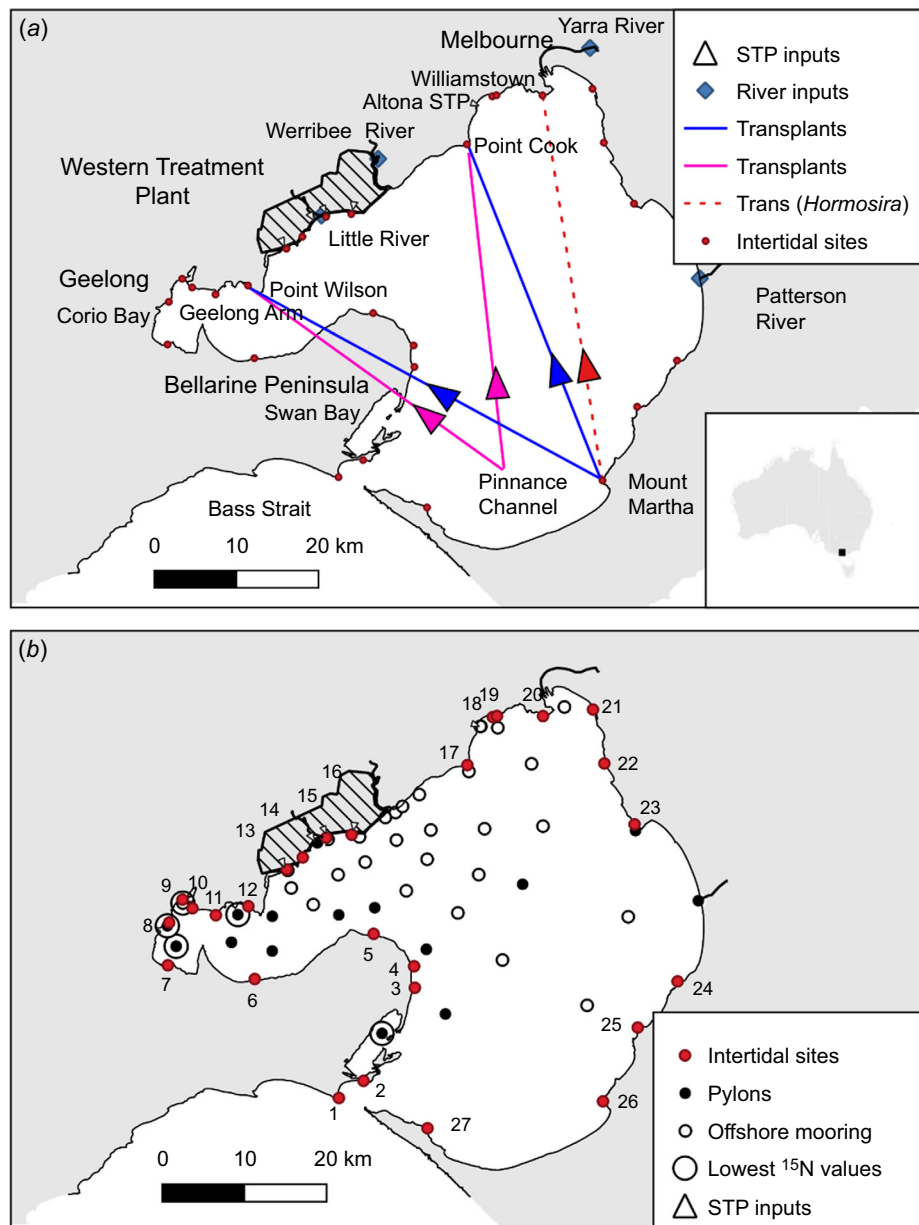


Fig. 1. Sampling sites in Port Phillip Bay. (a) Location of the STP inputs and of sampling sites on river inputs. The locations of all transplant sites are shown, including Williamstown, where only *Hormosira* was transplanted. Arrows indicate direction of transplantation. (b) Offshore sites (○) where *Botryocladia* and *Mytilus* were deployed, including offshore sites adjacent to pylons with *Mytilus* attached (●). Numbered sites (red) show where $\delta^{15}\text{N}$ was measured for intertidal species. Intertidal sites: 1. Point Lonsdale; 2. Queenscliff pilots pier; 3. St Leonards pier; 4. Indented Head; 5. Portarlington; 6. Avila Road; 7. Eastern Beach; 8. Pivot; 9. Geelong Grammar; 10. West of Avalon Beach; 11. Point Lillias; 12. Point Wilson quarry; 13. Murtcaim outlet; 14. Kirk Point; 15. 145 West outlet; 16. 15 East outlet; 17. Point Cook; 18. Altona pier; 19. North of Altona pier; 20. Williamstown; 21. St Kilda pier; 22. Brighton; 23. Ricketts Point; 24. Davey Bay; 25. Snapper Point; 26. Mount Martha; 27. Sorrento (Collins Settlement). Five larger circles show the five moorings or pylons with the lowest $\delta^{15}\text{N}$ values.

syringe filters, then frozen until analysed. Nutrient samples were analysed by the Water Studies Centre, Monash University. $\delta^{15}\text{N}$ of NO_3^- and NH_4^+ were analysed at Colorado Plateau Stable Isotope Laboratory (CPSIL), Northern Arizona

University (Parry 2013). Nutrient samples with salinities of $>10\text{‰}$ were excluded from the estimates to ensure only undiluted nutrient and $\delta^{15}\text{N}$ inputs were measured. This threshold was chosen on the basis of plots of the relationships

Table 1. Frequency of measurements at sampling sites between 3–5 July and 2–3 September 2012 (while biota were deployed on offshore moorings) of (a) salinity, nutrients (NH_4 , NO_x , PO_4) and $\delta^{15}\text{N}$ in NH_4 and NO_x of inputs to Port Phillip Bay, and (b) salinity and nutrients at intertidal and offshore sampling sites. (c) Sampling dates of $\delta^{15}\text{N}$ of intertidal and offshore biota between September 2011 and September 2012.

(a)	
Sites	Frequency
15 East, 145 West, Lake Borrie, Murtcaim, Little River, Werribee River, Yarra River	Weekly
Patterson River	Monthly
Altona STP	Once (August only)
(b)	
Sites	Frequency
Intertidal sites 1, 5–8, 12, 14, 17–18, 20	Weekly
Offshore sites 1–42	Monthly
(c)	
Sites	Sampling dates
Intertidal biota (<i>Capreolia</i> , <i>Hormosira</i> , <i>Galeolaria</i> and <i>Mytilus</i>)	
Sites 1–27	9–15 September 2012
Sites 1, 3–12, 14 and 17	9–15 September 2011 ^A , 12–18 March 2012, 19–30 June 2012
Offshore biota (<i>Botryocladia</i> and <i>Mytilus</i> , including <i>Mytilus</i> on pylons)	
Sites 1–42	2–3 September 2012

^A*Capreolia* only.

between nutrient concentrations versus salinity and salinity versus the difference between sampling time and low tide (Parry 2013).

During the field study, the nine major discharges to the Bay exhibited elevated mean concentrations of nutrients and $\delta^{15}\text{N}$

of NH_4^+ and NO_x (Table 2). The largest dissolved inorganic nitrogen (DIN) inputs to Port Phillip Bay came from the 15 East outlet of the WTP (10.6 tonnes N day⁻¹), the Yarra River (4.1 tonnes N day⁻¹), 145 West outlet of the WTP (1.7 tonnes N day⁻¹) and the Werribee River (0.83 tonnes N day⁻¹). All other inputs were less than 100 kg N day⁻¹ (Table 2). Notably, WTP discharges more NH_4^+ than NO_x , while rivers and drains are dominated by NO_x .

The values of $\delta^{15}\text{N}$ in DIN were two to three times higher at the WTP discharges (17–32‰) than in the Yarra River (8‰) and Werribee River (13‰, Table 2). Discharge from Altona STP was mid-range, with $\delta^{15}\text{N}$ = 15‰, but flows were small (Table 2).

Spatial distribution of $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ in biota

The red alga *Botryocladia sonderi* and the mussel *Mytilus galloprovincialis* were deployed in early July 2012 on moorings throughout Port Phillip Bay. Two months later in early September (Table 1C), $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ (algae only) were measured for these two offshore species and for four intertidal species.

The intertidal species were the algae, *Hormosira banksia* and *Capreolia implexa*, the filter-feeding serpulid worm, *Galeolaria caespitosa*, and the (intertidal or shallow subtidal) filter-feeding mussel, *Mytilus galloprovincialis*. In total, 27 sites around the perimeter of the bay were monitored (Fig. 1). Seasonal changes in $\delta^{15}\text{N}$ were also measured for these four species at 15 sites on the west of the bay (Table 1C).

The offshore species *Botryocladia* and *Mytilus* were deployed on the same 42 moorings. Moorings were located throughout the bay and 15 were deployed 50 m from channel markers where *Mytilus* was also sampled *in situ* (Fig. 1). Four

Table 2. Mean flow rates, ammonium and nitrate concentrations, $\delta^{15}\text{N}$ in ammonium and $\delta^{15}\text{N}$ in nitrate, and estimated nitrogen loads for nine main inputs to Port Phillip Bay, between July and September 2012.

Site	Load N (tonnes N day ⁻¹)															Mean (concentration-weighted) δ ¹⁵ N
	Flow (ML day ⁻¹) A		[NH ₄ ⁺] (μM) B		[NO ₃ ⁻] (μM) C		As NH ₄ ⁺ (A × B)		As NO ₃ ⁻ (A × C)		As DIN	δ ¹⁵ N-NH ₄ ⁺		δ ¹⁵ N-NO ₃ ⁻		
	Mean	s.e.	Mean	s.e.	Mean	s.e.	Mean	s.e.	Mean	s.e.		Mean	s.e.	Mean	s.e.	
Altona STP	9.8	0.2	126.2	20.7	510.7	46.2	0.02	0.003	0.07	0.008	0.09	33	0.5	10.9	0.4	15.3
Werribee River	404.4	97.5	5.4	0.9	141.7	20.7	0.03	0.012	0.80	0.311	0.83	28.9	5.1	12.4	0.7	13.0
15 East	456.1	7.7	757.1	73.6	910.7	55.2	4.83	0.552	5.82	0.451	10.65	19.2	2.9	15.9	0.6	17.4
145 West	75.4	7.6	1476.5	215.3	155.1	18.8	1.56	0.384	0.16	0.036	1.72	25.2	1.6	16.1	2	24.3
Little River	387.8	97.4	7		23.6		0.04		0.13		0.17	13.2		6.1		7.7
Lake Borrie	22.1	2.6	13.9	1.5	293.7	33.1	0.00	0.001	0.09	0.021	0.10	33.2	1.8	32	0.4	32.1
Murtcaim	1	0.1	10.8	1	81.4	13.5	0.00	0.000	0.00	0.000	0.00	22.9	2	24.3	2.2	24.1
Yarra River (Princes Bridge)	5632.4	251.7	3.7	0.3	48	1.2	0.29	0.037	3.78	0.264	4.08	20.3	7.4	7.1	0.2	8.0
Patterson River			3.5	1.2	73.8	13.5						14.8		10.6	0.4	

Standard errors are shown for all estimates. The mean load-weighted $\delta^{15}\text{N}$ for each input is shown.

moorings, two in Corio Bay and two in the east of the bay, were lost.

Offshore moorings consisted of rope held on the seabed with a steel weight connected to a 200-mm diameter polystyrene float 1–1.5 m below the sea surface and a small surface buoy. *Botryocladia* and *Mytilus* were attached 0.5–1.0 m below the subsurface buoy. At each site, *Botryocladia* was deployed with two different methods. *Botryocladia* stems were inserted into 30 cm lengths of surgical rubber tubing and held there using small cable ties and it was also placed in an open-ended plastic cylinder enclosed by a mesh bag held tight by a cable tie (Parry 2013). Samples obtained from both deployment methods consisted of 4–12 newly grown (pink, shiny, clean and distal) vesicles that were dissected from each plant.

Mytilus was obtained from a mussel farm at Pinnacle Channel near the open-ocean entrance to the Bay (Fig. 1) and 20 were deployed in mesh oyster bags on each mooring.

Following collection, *Capreolia* was shaken in seawater to remove as much sand as possible, then any epiphytes and any substrate still attached to its holdfasts were carefully removed under a dissecting microscope. *Galeolaria* and *Mytilus* were dissected in the laboratory with fine scissors. At each site, opercular adductor muscles and feeding crowns were removed from 30 *Galeolaria* and a composite sample of each tissue was sampled and analysed. Similarly, at each site the posterior adductor muscle and a sample of gill tissue (approximately 1 cm × 1 cm) was dissected from five *Mytilus* individuals and a composite sample of each tissue was analysed. At each site, a sample of 10 penultimate *Hormosira* vesicles, with smaller newly grown distal vesicles attached, were collected. Two samples were analysed from each site. One consisted of old growth in the 10 larger (5–10 mm diameter) penultimate vesicles and the other consisted of new growth from all the attached (<5 mm diameter) distal vesicles, typically 15–20 small vesicles per sample.

All samples for $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ analysis were dried at 60°C for 24–48 h, then ground in a mortar and pestle. Values of $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ for algae and invertebrates were measured at the Water Studies Centre, Monash University.

Transplant experiments

Transplant experiments were conducted to determine the period over which *Capreolia*, *Hormosira*, *Galeolaria* and *Mytilus* integrate the ^{15}N signal by transferring them to higher-background ^{15}N sites. Changes in their $\delta^{15}\text{N}$ were monitored weekly initially, then at longer intervals, for 4–5 months. Between 9 and 12 June 2012, all four species were transplanted from areas of low ^{15}N (Mount Martha and Pinnacle Channel) to areas of high ^{15}N (Point Cook and Point Wilson). The latter sites were the closest intertidal reefs north and south of the WTP. In addition, *Hormosira* was transplanted to Williamstown (slightly north of Point Cook) because there was no *in situ* *Hormosira* at Point Cook, so

conditions there may not have been suitable for it. *Mytilus* was transplanted from a mussel farm at Pinnacle Channel and deployed on short 1-m moorings in water 1–1.5 m deep. Other taxa were transplanted from Mount Martha on rocks that were placed adjacent to the same taxa as those growing *in situ*, except where there was no *in situ* *Hormosira* at Point Cook.

Turnover of isotopes in tissue is usually approximated by an exponential relationship (McIntyre and Flecker 2006), as adopted here for transplanted *Galeolaria* and *Mytilus*. These exponential curves are of the form $y = a + be^{kt}$, where a , b and k are constants and t = time. The half-life of tissue turnover is given by $T_{1/2} = \log_e 2/k$, where e is the base for natural logarithms (Gustafson *et al.* 2007; Ankjær *et al.* 2012). Exponential curves were not fitted to describe changes in $\delta^{15}\text{N}$ of the algae *Capreolia* and *Hormosira* because most change in these species occurred in the week after their transplantation. For *Galeolaria*, changes after 3 months no longer fitted an exponential curve; hence, the curve was fitted excluding the last two sampling dates (Parry 2013).

Application of Bubbles NPZ Model

The three-dimensional nutrient–phytoplankton–zooplankton (NPZ) model *Bubbles* has been shown to provide close predictions of nutrients and planktonic productivity (Black *et al.* 2016; Jenkins and Black 2019, 2021). When calibrated against field data in Port Phillip Bay, the model predicted the concentration of the 12 most abundant phytoplankton, down to individual species and total diatoms and flagellates.

Most recently, *Bubbles* was calibrated over a 10-year period from 2010 to 2019 inclusive (Black *et al.* 2022). Calibration compared the model to monthly measurements from the EPA (Victoria) at six sites that encompass the main hydrodynamic and biological zones around Port Phillip Bay, from the lower currents of Corio Bay to the higher nutrient loads near WTP, the Yarra River, and the ocean-influenced Sands region at Dromana. The 10-year period experienced a range of wet and dry years. The model simulations are annual, starting from 1 July of each year. The model uses a cold start where key variables such as the initial N values are set from data at six sites interpolated onto all model cells. Consequently, the first 1–2 weeks of the simulation are still coming into balance with the actual baywide conditions, so the initial 2 weeks of model runs were excluded from the analyses in this paper.

The model was checked against 12 individual species of phytoplankton, total diatoms and total flagellates, nutrient concentrations, ratio of N species, seabed N and Si fluxes/concentrations, temperature, salinity and Chl-*a* for a total of 22 different parameters. The model output is written to a stand-alone file at 1 hourly intervals over the 10-year period. In this paper, the required data were extracted and analysed for the period of the ^{15}N experiments.

Model *Bubbles* uses output from a three-dimensional, stratified ocean/atmosphere hydrodynamic model to specify

the currents for advection and diffusion. *Bubbles* considers the N cycle via growth and decay of phytoplankton and zooplankton, immediate remineralisation, seabed denitrification and losses to the atmosphere.

The model was used to:

1. Estimate the footprint of WTP-sourced DIN, because direct measurements of DIN during the study were available only on three potentially unrepresentative days.
2. Predict the distribution of phytoplankton that takes up N sourced from the WTP to compare with $\delta^{15}\text{N}$ of mussels that feed on this phytoplankton.
3. Compare the measured $\delta^{15}\text{N}$ footprints of the WTP with the DIN and phytoplankton footprints of the WTP predicted by the model. Some differences between the model and $\delta^{15}\text{N}$ measurements were expected where isotope fractionation occurred during uptake of N or seabed denitrification, because these were not modelled.

Results

Nutrient measurements

Offshore water sampling was undertaken three times between July and September 2012. When combined with simultaneous intertidal sampling, these showed that NH_4^+ , NO_x and PO_4^{-3} concentrations were high along the north-western coast adjacent to and north of the WTP. NO_x was also high near the Patterson and Yarra rivers (Fig. 2), whereas the WTP was the dominant source of DIN. Concentrations of all nutrients declined offshore from the WTP, but NH_4^+ and NO_x declined more sharply than did PO_4^{-3} (Fig. 2).

Nutrient concentrations at the 10 intertidal sites showed the anticipated high values north of the WTP (Sites 14, 17, 18 and 20, Table 3, Fig. 3c). A secondary DIN peak occurred on the southern side of the Geelong Arm at Site 6 (Fig. 3c).

As a validation of the numerical model, Fig. 2d shows DIN ($\text{NO}_x + \text{NH}_4^+$) averaged over the experimental period from 14 July to 4 September 2012. DIN was measured on three occasions, always during calm conditions, when the wind was offshore from the north-western quadrant, whereas the model averages show all conditions at 1-hourly intervals over a 6-week period, when wind and plume dynamics differed widely (Fig. 4). Extreme plume fluctuations expose monitored species to WTP effluent where N enriched in ^{15}N will be taken up, but they do not dominate the 2-month mean. Both the patterns and magnitudes in the model (Fig. 2d) are very similar to the NH_4^+ and NO_x measurements (Fig. 2a, b). Key features include (I) high concentrations near the shoreline around the WTP outfalls; (II) bulge to the south-east towards Bellarine Peninsula (as seen in the NH_4^+ measurements); (III) low concentrations between Altona and the Yarra River; and (IV) plumes associated with the Yarra and Patterson rivers.

Differences in $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ owing to season, tissue type and deployment method

Temporal differences in $\delta^{15}\text{N}$ were small in all species compared with spatial differences, and showed no clear seasonal pattern, except that $\delta^{15}\text{N}$ for *Galeolaria* was consistently 0.5–1.5‰ lower at all sites in March 2012 (Supplementary Fig. S1). Similarly, differences in $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ between new and old growth of *Hormosira*, and between $\delta^{15}\text{N}$ from different tissues of *Galeolaria* and *Mytilus* were small compared with spatial differences, and all showed very similar spatial patterns for *in situ* specimens (Fig. S2) and similar temporal patterns for transplanted specimens (Fig. S3). Notably, muscle tissue from transplanted *Galeolaria* and *Mytilus* adopted the $\delta^{15}\text{N}$ of their new site more slowly than did their crowns and gills (Fig. S3). In all subsequent analyses, at each site measurements from the three main sampling times (March 2012, June 2012 and September 2012), new and old growth of *Hormosira*, and both tissue types for *Galeolaria* and *Mytilus*, were averaged.

Botryocladia had more epiphytes when attached to moorings by surgical tubing rather than in (more shaded) plastic cylinders (Parry 2013). The value of $\delta^{15}\text{N}$ of *Botryocladia* was slightly higher in tubes than in plastic cylinders (Fig. S4). Because both replicates of *Botryocladia* in tubes were lost from some moorings, mean $\delta^{15}\text{N}$ of *Botryocladia* at each site was estimated using all replicates (tubes and cylinders), after making a small correction for differences in $\delta^{15}\text{N}$ caused by different deployment techniques, by using the equation shown in Fig. S4. There was no significant difference in $\delta^{13}\text{C}$ between *Botryocladia* deployed in tubes and those in cylinders.

Spatial distribution of $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ in biota

Intertidal biota

For the intertidal species, there were substantial and highly correlated spatial differences in $\delta^{15}\text{N}$ of *Capreolia*, *Hormosira*, *Galeolaria* and *Mytilus*. Low values were found for all species south and west of the WTP (Sites 1–12, Fig. 3a), whereas very high values occurred adjacent to and north of the WTP (Sites 13–17, Fig. 3a) and these gradually diminished clockwise around the bay (Sites 18–27, Fig. 3a). However, $\delta^{15}\text{N}$ values for all species were higher along the eastern coast than in the Geelong Arm.

Similar spatial differences among $\delta^{15}\text{N}$ signatures of filter feeders, *Galeolaria* and *Mytilus* were shown in all seasons, and although spatial differences in $\delta^{15}\text{N}$ signatures in the macroalgae *Capreolia* and *Hormosira* were also similar, they were partially masked by greater temporal variation (Fig. S1). At Site 1, distant from WTP discharges, $\delta^{15}\text{N}$ signatures of *Capreolia* and *Hormosira* (range 2.5–6.1‰) were similar to that of NO_3^- in oxic deep-ocean water (3–6‰, mean 4.8‰, Montoya 2007).

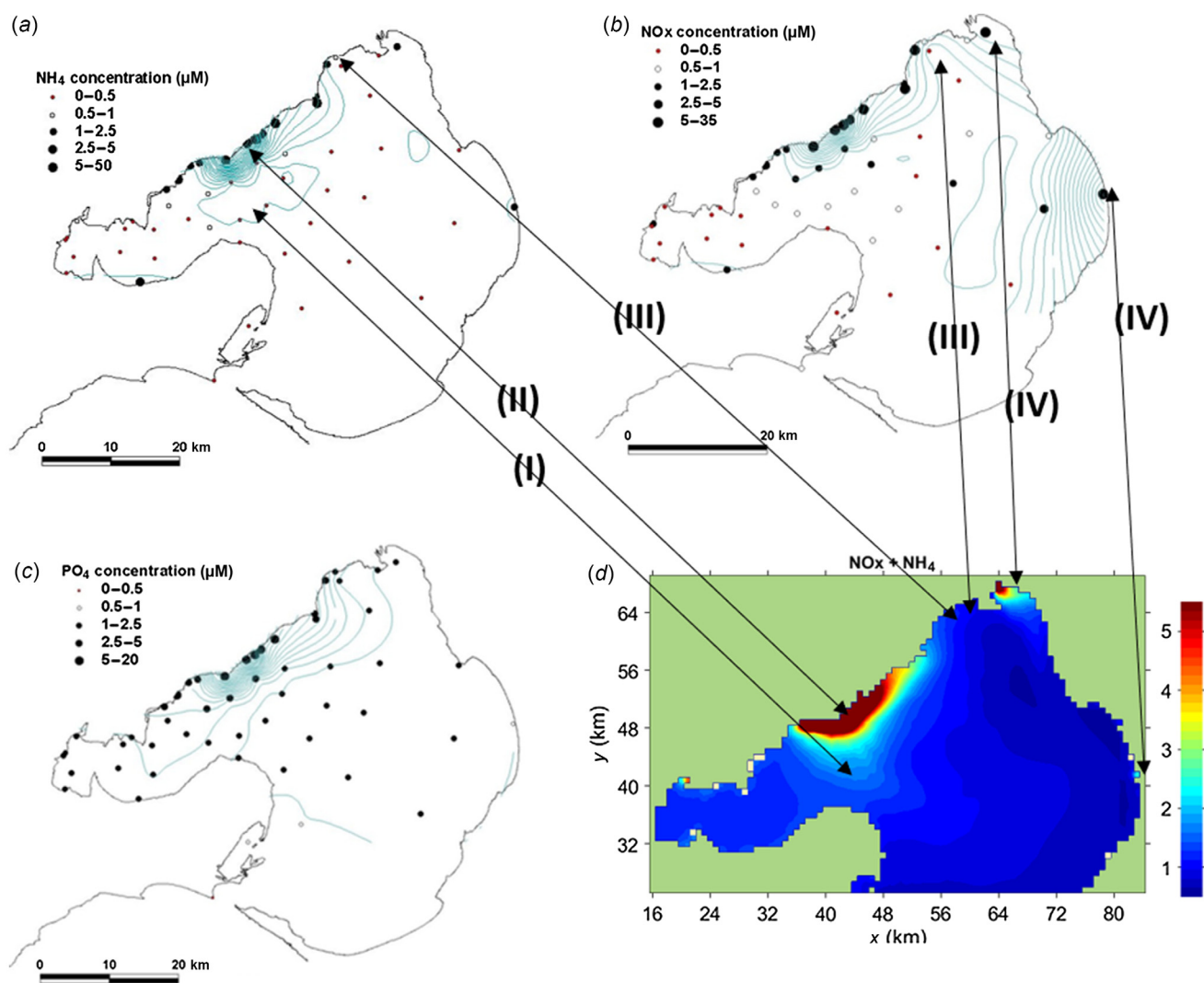


Fig. 2. Mean nutrient concentrations based on onshore ($N = 8$) and offshore ($N = 3$) measurements between June and September 2012 for (a) NH_4^+ , (b) NO_x and (c) PO_4^{3-} . (d) Output from *Bubbles* model for $\text{NH}_4^+ + \text{NO}_x$ (μM) in the period 14 July 2012 to 4 September 2012. The arrows show the key similarities, namely (I) bulge in plume towards Bellarine Peninsula, (II) high concentration around WTP, (III) low concentrations to the west of the Yarra River near Altona, (IV) Yarra River and Patterson River plumes.

Table 3. Nutrient concentrations (mean and standard errors) at intertidal sites monitored between July and September 2012.

Site	Site code	N	Total DIN (μM)		NO $_x$ (μM)		NH $_4$ (μM)		PO $_4$ (μM)	
			Mean	s.e.	Mean	s.e.	Mean	s.e.	Mean	s.e.
Point Lonsdale	1	8	0.95	0.16	0.78	0.16	0.17	0.05	0.27	0.01
Portarlington	5	8	0.41	0.10	0.23	0.06	0.18	0.05	1.65	0.09
Avila Road	6	8	4.43	2.62	1.01	0.26	3.42	2.41	1.33	0.22
Eastern Beach	7	8	0.51	0.14	0.21	0.05	0.30	0.12	1.76	0.04
Pivot	8	8	1.76	0.57	1.33	0.53	0.44	0.10	2.00	0.08
Point Wilson quarry	12	8	0.61	0.29	0.33	0.18	0.29	0.12	1.80	0.11
Kirk Point	14	8	9.60	4.13	3.79	1.85	5.80	2.77	5.51	0.88
Point Cook	17	8	3.63	1.11	2.07	0.99	1.56	0.28	2.66	0.12
Altona pier	18	8	1.65	0.43	0.86	0.30	0.79	0.17	1.92	0.16
Williamstown	20	8	1.41	0.30	0.92	0.29	0.49	0.03	1.79	0.07

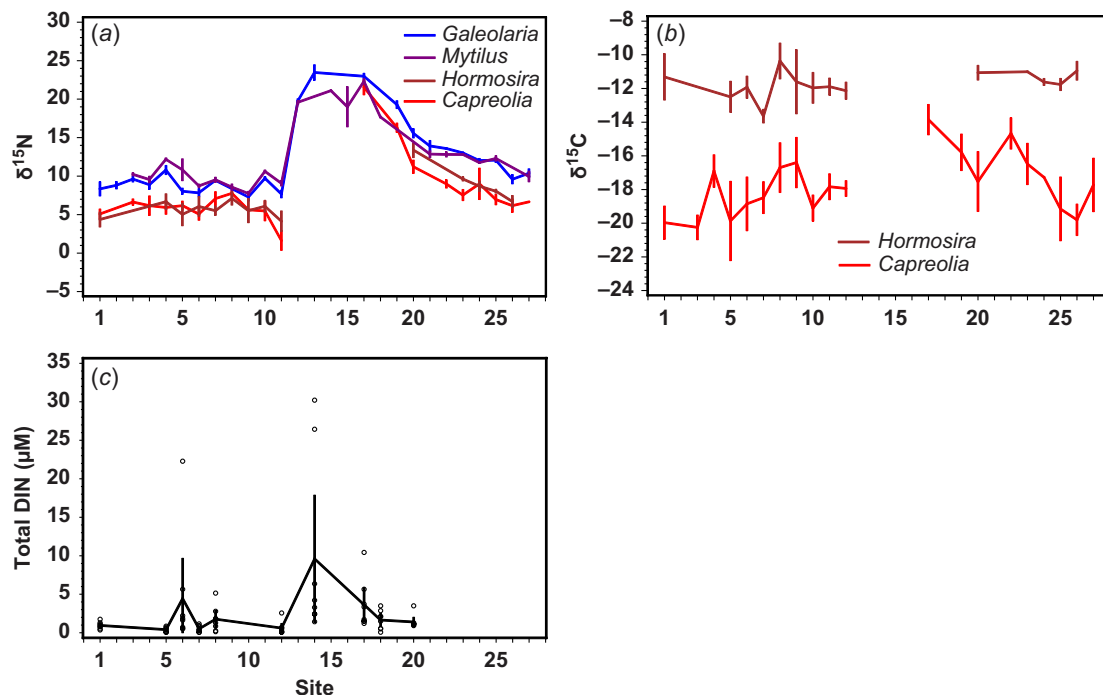


Fig. 3. Spatial differences at Sites 1–27 (see Fig. 1) in (a) $\delta^{15}\text{N}$ of four intertidal species growing *in situ*, (b) $\delta^{13}\text{C}$ of two intertidal algae growing *in situ*, and (c) DIN ($\text{NH}_4 + \text{NO}_x$) (μM), on the basis of measurements between June and September 2012. All error bars are standard errors.

In the Geelong Arm (Sites 5–12, Fig. 1), differences in $\delta^{15}\text{N}$ of *Galeolaria* and *Mytilus* were evident at a small spatial scale. Values were higher at Sites 5 and 11 than at sites immediately adjacent to them (Fig. 3a). The lowest $\delta^{15}\text{N}$ values for *Capreolia* and *Hormosira* occurred at Site 12 (Point Wilson quarry) (mean $\delta^{15}\text{N} = 1.6\text{‰}$ and 4.1‰ , respectively, Fig. 3a), where the highest within-site variation also occurred (Fig. S1).

There was minimal difference in $\delta^{15}\text{N}$ between *in situ* filter-feeders *Galeolaria* and *Mytilus* ($0.34 \pm 0.23\text{‰}$ s.e.) and between the *in situ* algae *Capreolia* and *Hormosira* ($0.37\text{‰} \pm 0.30\text{‰}$ s.e.) across all sites at which they co-occurred. The mean difference in $\delta^{15}\text{N}$ between co-occurring plants and filter-feeders was 3.2–3.8‰.

Spatial differences in $\delta^{13}\text{C}$ (Fig. 3b) were similar to those seen in $\delta^{15}\text{N}$ (Fig. 3a), except that there was more variation that was unrelated to its proximity to the WTP. For example, high $\delta^{13}\text{C}$ values for *Capreolia* occurred not only near the WTP, but also at Sites 4, 22 and 23 far from the WTP. The value of $\delta^{13}\text{C}$ of *Hormosira* was much higher than that for *Capreolia*, and showed minimal spatial variation, but this species does not occur between Sites 12 and 20, where DIN is high. The value of $\delta^{13}\text{C}$ of *Capreolia* was highest at Point Cook (Site 17, Fig. 3b), the closest site north of the WTP where it occurred.

Offshore biota

At the offshore sites, the general pattern in Fig. 4a–c is a broad 10–15 km wide zone off the WTP of high DIN, high

$\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ for *Botryocladia* and high $\delta^{15}\text{N}$ for *Mytilus*. This zone extends north-west to the Yarra River, whereas its width and magnitude both reduce northward. The zone extends into the Geelong Arm immediately south of the WTP but does not usually extend west of Point Wilson. Some notable differences among patterns shown by DIN, $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ include the following:

1. DIN concentrations had a smaller footprint than did $\delta^{15}\text{N}$ for *Botryocladia* and *Mytilus*, on the basis of a comparison of Figs 2 and 5, which share the same sampling sites.
2. $\delta^{15}\text{N}$ values for *Botryocladia* declined progressively offshore, but high values nearest the outfalls may not reflect the very high DIN concentrations measured there. For example, the mean $\delta^{15}\text{N}$ measured in the 145W discharge was 24.3‰ (Table 2), but $\delta^{15}\text{N}$ of the closest *Botryocladia*, deployed 500 m away, was 18.5‰ .
3. $\delta^{15}\text{N}$ values for *Mytilus* showed even less variation across the width of the plume than did those for *Botryocladia*.
4. Modelled distributions of DIN showed spatial patterns similar to those of $\delta^{15}\text{N}$ of *Botryocladia* and modelled flagellate distributions mirrored $\delta^{15}\text{N}$ of phytoplankton consuming *Mytilus*, except possibly in Hobsons Bay.
5. Two adjacent sites offshore from the north-east of the Bellarine Peninsula had high and low values for $\delta^{13}\text{C}$ for *Botryocladia* that were unrelated to their proximity to the WTP.

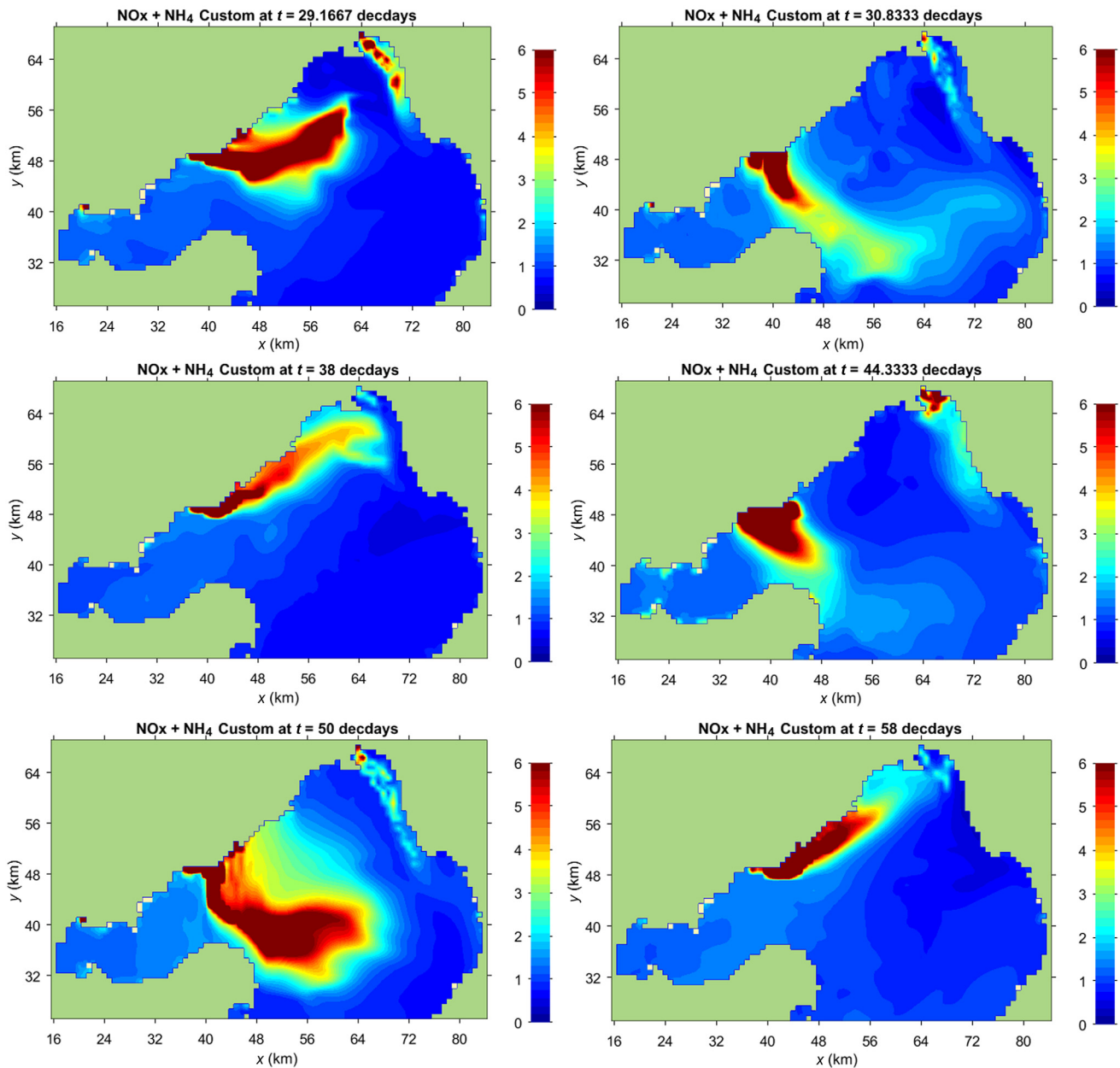


Fig. 4. Examples of $\text{NH}_4^+ + \text{NOx}$ (μM) plume dynamics during different wind conditions between 3 July and 4 September 2012.

The five sites with the lowest $\delta^{15}\text{N}$ of *Botryocladia* and *Mytilus* were in Swan Bay, three sites in Corio Bay and along the northern coastline between Corio Bay and WTP (Figs 1b, 5a, c, d).

The $\delta^{15}\text{N}$ of *Mytilus* collected *in situ* on pylons showed a spatial pattern similar to that of *Mytilus* deployed on moorings 50 m away (Fig. 4c, d). The values of $\delta^{15}\text{N}$ of *Mytilus* on 2-month deployments on moorings converged towards values of $\delta^{15}\text{N}$ of *Mytilus* that had lived on adjacent pylons for their lifetime (Fig. 6).

Although the WTP was the major influence on spatial patterns of $\delta^{15}\text{N}$ in Port Phillip Bay, there were several minor influences. The low $\delta^{15}\text{N}$ of the Werribee River ($\delta^{15}\text{N}$

of $\text{DIN} = 13\text{‰}$), which discharges within the WTP zone ($\delta^{15}\text{N}$ of DIN at 15 East outlet = 17‰), appeared to cause a region of lower $\delta^{15}\text{N}$ adjacent to the coast for both *Botryocladia* and *Mytilus* north and south of the Werribee River (Fig. 5a, c). The footprint of the small Altona STP ($<100 \text{ kg N day}$) probably declined sharply with distance from the outlet, but its apparent footprint shown by contours in Fig. 5a was exaggerated by a lack of nearby sampling stations.

Transplant experiments

The values of $\delta^{15}\text{N}$ of *Capreolia*, *Hormosira*, *Galeolaria* and *Mytilus* transplanted to Point Cook and Williamstown

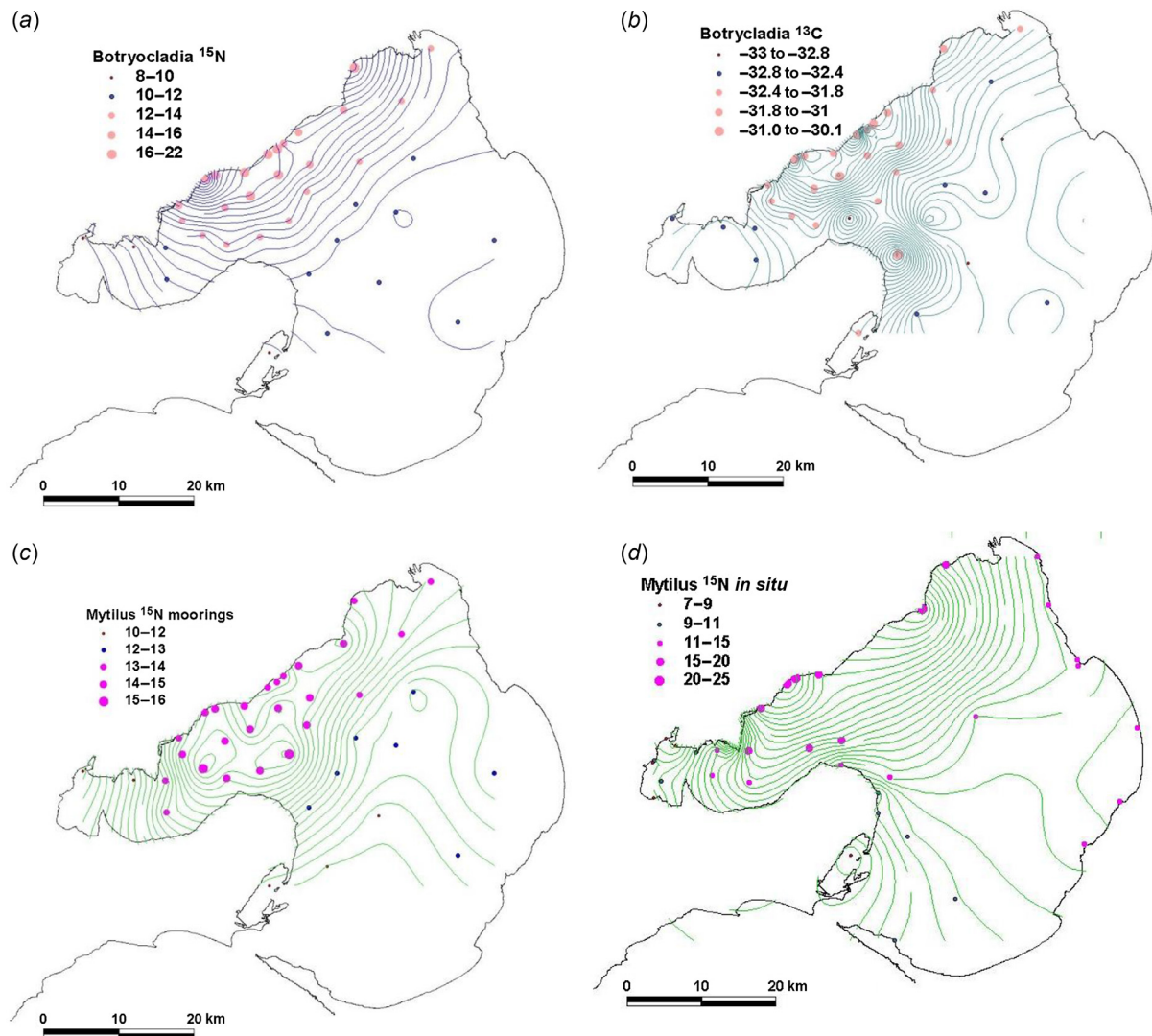


Fig. 5. Spatial variation during September 2012 in species deployed on offshore moorings between June and September 2012. (a) $\delta^{15}\text{N}$ in *Botryocladia*, (b) $\delta^{13}\text{C}$ in *Botryocladia* (c) $\delta^{15}\text{N}$ in *Mytilus* and (d) collected *in situ* on pylons $\delta^{15}\text{N}$ in *Mytilus* in September 2012.

increased, but biota transplanted to Point Wilson quarry did not because, despite its proximity to the WTP, $\delta^{15}\text{N}$ values of *in situ* biota there were similar to those at Mount Martha and Pinnacle Channel (Figs 1a, 6).

At the transplant sites, $\delta^{15}\text{N}$ of control/*in situ* *Galeolaria* and *Mytilus* showed little change during the 3–5 month transplant experiment, whereas $\delta^{15}\text{N}$ of control/*in situ* algae *Capreolia* and *Hormosira* varied (Fig. 7).

Following transplantation to Point Cook, the rate that $\delta^{15}\text{N}$ increased varied greatly among species. The rate of change for *Capreolia* and *Hormosira* was much greater in the week following their transplantation than subsequently (Fig. 7). The value of $\delta^{15}\text{N}$ of *Capreolia* increased for ~6–12 weeks when an asymptote appeared to be reached (Fig. 7), but there was a temporal trend in $\delta^{15}\text{N}$ of *in situ*/control *Capreolia*, making it difficult to determine precisely when/if the

transplanted and *in situ* measurements converged (Fig. 7). The value of $\delta^{15}\text{N}$ of *Hormosira* transplanted to Point Cook and Williamstown approached asymptotes after ~10 weeks, when $\delta^{15}\text{N}$ of *Hormosira* transplanted to Williamstown also converged with *in situ* values, although there was substantial temporal variation in the latter (Fig. 7).

Values of $\delta^{15}\text{N}$ of *Galeolaria* transplanted to Point Cook increased slowly for ~2.5 months, when an asymptote may have been reached, but at a lower value than for *in situ* samples (Fig. 7). An exponential curve describing changes in $\delta^{15}\text{N}$ of *Galeolaria* for the first 2.5 months is as follows:

$$\text{Log}_e(\delta^{15}\text{N}_t/\delta^{15}\text{N}_0) = 0.821 + 0.00769 \times \text{days}, \text{ and the}$$

half-life of tissue turnover for *Galeolaria* was 90 days ($= \log_e 2/0.00769$).

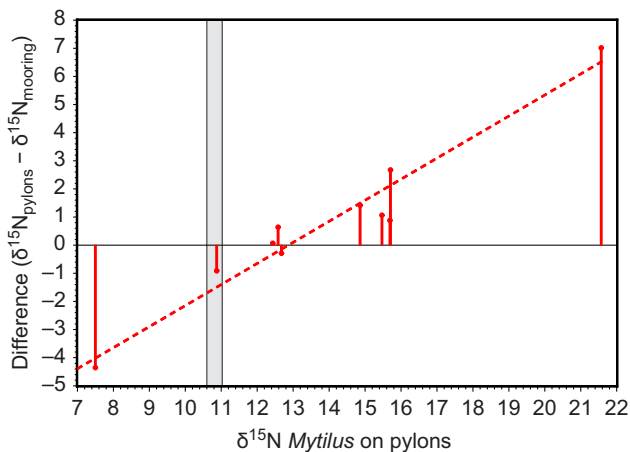


Fig. 6. Plot of ($\delta^{15}\text{N}$ of *Mytilus* growing in situ on pylons – $\delta^{15}\text{N}$ of *Mytilus* deployed on moorings 50 m away) against $\delta^{15}\text{N}$ of *Mytilus* growing in situ on pylons. Measurements were obtained in September 2012. The grey area is $\delta^{15}\text{N}$ of *Mytilus* on deployment, based on measurements in June and September 2012 at Pinnacle Channel, where all deployed mussels were collected.

Values of $\delta^{15}\text{N}$ of *Mytilus* transplanted to Point Cook increased slowly for the 4 month duration of the transplant experiment (Fig. 7). At this time, $\delta^{15}\text{N}$ values of transplanted *Mytilus* were still much lower than $\delta^{15}\text{N}$ values of *in situ* *Mytilus* (Fig. 7). An exponential curve describing changes in $\delta^{15}\text{N}$ of *Mytilus* for the 4 months of transplantation is as follows:

$$\text{Log}_e(\delta^{15}\text{N}_t/\delta^{15}\text{N}_0) = -0.0129 + 0.00295 \times \text{days}, \text{ and the} \\ \text{half-life of tissue turnover for } \textit{Mytilus} \\ \text{was } 234 \text{ days } (= \log_e 2/0.00295).$$

Discussion

The nutrient footprint of the WTP

DIN measurements, modelled DIN plumes and stable isotope ratios in *Botryocladia* and *Mytilus* all broadly indicate that the footprint of WTP-sourced N extends 10–15 km offshore from the WTP along the north-western shoreline, extending into the Geelong Arm immediately south of the WTP, but does not usually affect the Geelong Arm west of Point Wilson or the eastern two-thirds of Port Phillip Bay. The footprint of $\delta^{15}\text{N}$ of *Mytilus* extended ~10–15 km offshore, with the extension being slightly larger than that of *Botryocladia* (~10 km), because *Mytilus* consumes phytoplankton that disperse after taking up WTP-sourced N.

Measured and modelled DIN concentrations and $\delta^{15}\text{N}$ of *Botryocladia* showed similar spatial patterns (compare Figs 2 and 4a). The pattern of $\delta^{15}\text{N}$ of *Botryocladia* should reflect that of the N-enriched plume of the WTP, because it obtains its N from the water column directly. The model shows that

the WTP plume is very variable and highly dependent on wind conditions (Fig. 4). Although short-lived events do not dominate the 2-month mean, fluctuations in the plume still expose monitored species to WTP effluent and allow ^{15}N to be taken up over a broad region that will vary with seasonal winds. The modelled DIN plume periodically impinges on the coast of the northern Bellarine Peninsula (Fig. 4), explaining the high $\delta^{15}\text{N}$ values of *Galeolaria* and *Mytilus* at Site 5 (Fig. 3a).

There were important deviations from the broadly similar patterns shown by DIN, $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$. Several of these deviations are likely to be due to fractionation on uptake of N. Fractionation results from biological processes preferentially consuming the lighter N isotope (^{14}N), resulting in the residual reactant pool becoming enriched in the heavier isotope (^{15}N) (Kendall *et al.* 2007; Montoya 2008).

Typically, fractionation causes $\delta^{15}\text{N}$ to increase by 3–4‰ with each trophic level (Peterson and Fry 1987; Post 2002; Jennings and Warr 2003), being similar to the mean difference in $\delta^{15}\text{N}$ between co-occurring intertidal plants and filter-feeders (3.2–3.8‰).

Fractionation owing to mineralisation of organic matter is consistently near zero (Brandes and Devol 1997; Montoya 2008), whereas fractionation by nitrifying bacteria in sediments typically causes isotope effects of 15–25‰, and by denitrifying bacteria of 20–30‰ (Montoya 2008). However, the isotope effect across the sediment water interface (ϵ_{sed}) is often near zero, because nitrification and denitrification are limited by the available N (Brandes and Devol 1997; Lehmann *et al.* 2007; Devol 2015; Montoya 2008; Glibert *et al.* 2019). In addition, nitrification and denitrification have compensatory effects on ϵ_{sed} because the light NO_3 from nitrification becomes the input to denitrification that creates heavier NO_3 and light N_2 (Devol 2008). The value of ϵ_{sed} is closest to zero at the most oxygenated sites with the minimal reactive organic matter (Lehmann *et al.* 2004; Alkhatib *et al.* 2012), conditions similar to those found in most sediments in Port Phillip Bay, whereas ϵ_{sed} is most likely to be greater than 0‰ where denitrification efficiency is low and surface sediments are poorly oxygenated (Lehmann *et al.* 2004; Granger *et al.* 2011; Alkhatib *et al.* 2012; Kessler *et al.* 2014).

Fractionation owing to uptake of N by algae is often dependent on DIN concentration (Swart *et al.* 2014). ^{15}N is detected by *Botryocladia* with great sensitivity near the edge of the plume, but nearshore where DIN is high, $\delta^{15}\text{N}$ values may be damped by preferential uptake of ^{14}N . The very high concentrations of DIN and very high $\delta^{15}\text{N}$ values in WTP discharges were not mirrored by very high $\delta^{15}\text{N}$ values in *Botryocladia* nearest the WTP. This difference is likely to be due to fractionation on N uptake. *Botryocladia*, like other plants (Dudley *et al.* 2010; Swart *et al.* 2014; Orlandi *et al.* 2017; Pruell *et al.* 2020), probably takes up ^{14}N preferentially at high DIN concentrations, whereas at low DIN concentrations near the edge of the plume, all N is taken up and there is no fractionation. Swart *et al.* (2014) found that

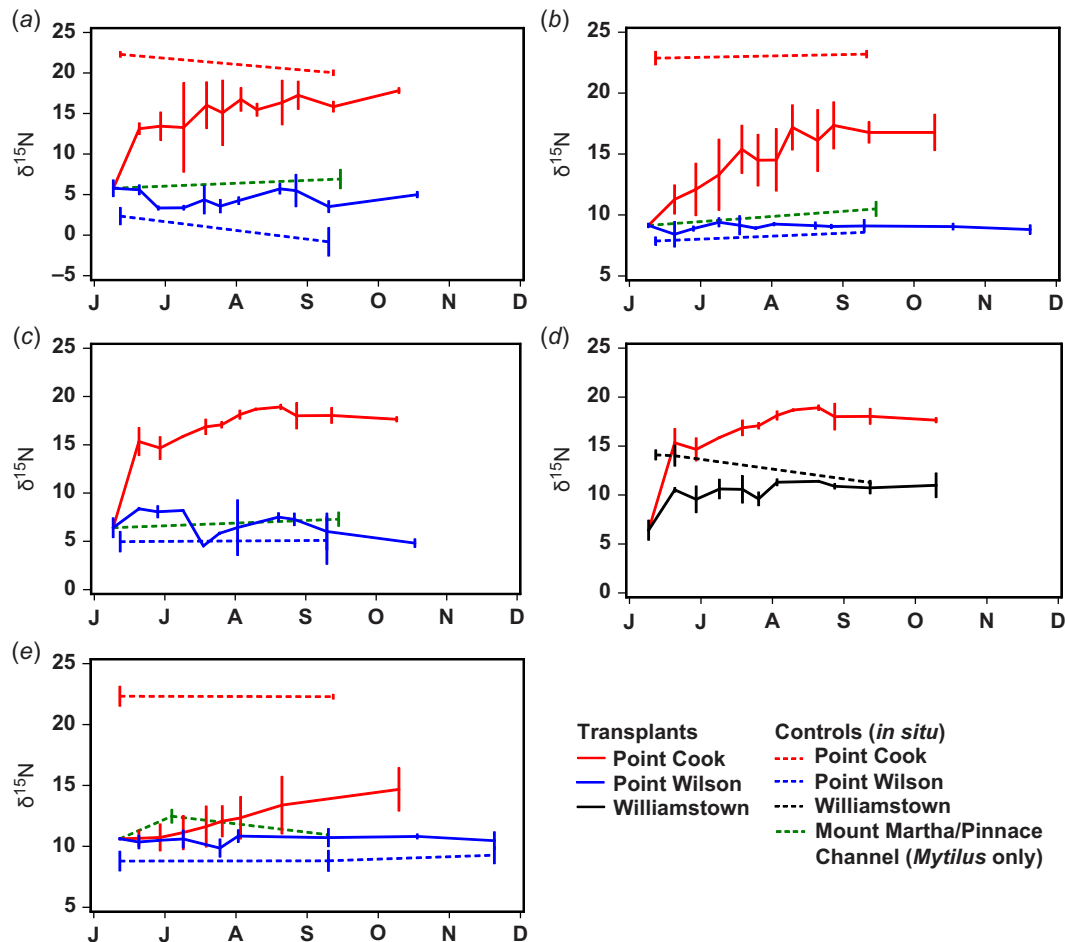


Fig. 7. Comparison of changes in $\delta^{15}\text{N}$ of *in situ* and transplanted species between June and December 2012 (months are indicated by their first letters). (a) *Caprellaia* transplanted from Mount Martha to Point Cook and Point Wilson, (b) *Galeolaria* transplanted from Mount Martha to Point Cook and Point Wilson, (c) *Hormosira* transplanted from Mount Martha to Point Cook and Point Wilson, (d) *Hormosira* transplanted from Mount Martha to Point Cook and Williamstown, (e) *Mytilus* transplanted from Pinnacle Channel to Point Cook and Point Wilson.

fractionation of nitrate by macroalgae ceased when its concentration was $\sim 2 \mu\text{M}$.

Minimal variation shown in $\delta^{15}\text{N}$ of *Mytilus* across the width of the WTP plume is also probably the result of fractionation. In contrast to fractionation shown by algae, filter-feeders (*Daphnia*) fed algae with high N content showed near-zero fractionation, whereas those fed algae with low N showed fractionation of $\sim 6\text{‰}$, possibly owing to greater excretion of ^{14}N when N intake is lower (Adams and Sterner 2000). Similarly, an experimental study found that phytoplankton continuously cultured in high DIN concentrations showed a depletion of $\delta^{15}\text{N}$ of up to 9‰ compared with algae cultured in low DIN, but $\delta^{15}\text{N}$ of mussels feeding on phytoplankton from high DIN treatments had $\delta^{15}\text{N}$ values only 2‰ lower than those on feeding on low DIN phytoplankton (Pruell *et al.* 2020). A conceptual model of fractionation near the WTP, on the basis of Adams and Sterner (2000), across two trophic levels in areas of high and low DIN, is shown in Fig. 8.

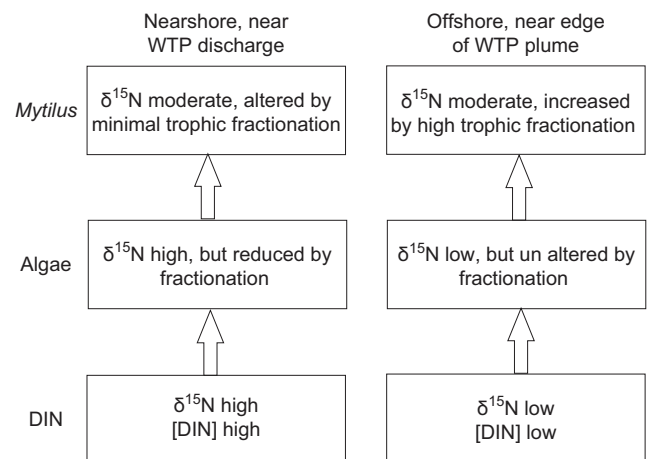


Fig. 8. Conceptual diagram showing fractionation processes at two trophic levels near (high DIN) and distant from the WTP (low DIN), based on Adams and Sterner (2000).

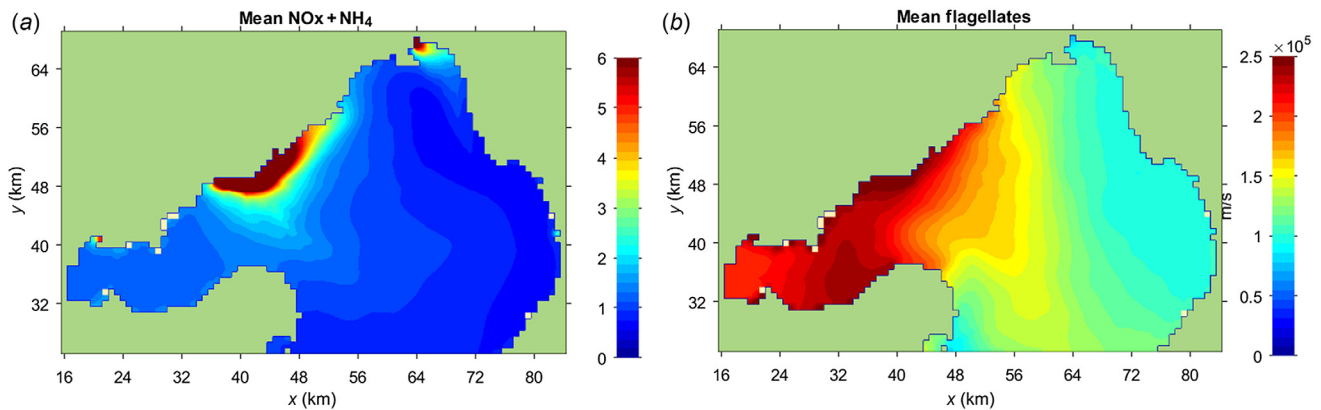


Fig. 9. Outputs from the *Bubbles* model (Jenkins and Black 2021) for the period 14 July to 3 September 2012, when *Botryocladia* and *Mytilus* were deployed in the bay, showing (a) $\text{NO}_x + \text{NH}_4^+$ concentration (μM) in Port Phillip Bay, and (b) average flagellate concentration ($\text{individuals.L}^{-1}$).

The *Bubbles* model incorporates N utilisation, recycling and eventual losses to reproduce the DIN plume. For example, the salinity averaged over the same 3 months shows no significant correspondence with the distribution of NH_4^+ or flagellates (compare Figs 9 and S5). Salinity is a conservative tracer, not subject to the complexities of the N cycle. Similarly, PO_4^{3-} shows a much more even distribution than does NH_4^+ (Fig. 2) because P is abundant in the bay, and studies have shown that N, rather than P, is the limiting nutrient (Harris *et al.* 1996) for flagellates.

The *Bubbles* model of DIN concentrations (Figs 2d, 9a) closely predicts the patterns shown by $\delta^{15}\text{N}$ in *Botryocladia* (Fig. 4a), and the predicted flagellate distribution (Fig. 9b) closely mirrors the patterns shown by $\delta^{15}\text{N}$ in the phytoplankton consuming *Mytilus* (Fig. 4c). The latter has the larger footprint because phytoplankton disperse after consuming WTP-sourced N.

The values of $\delta^{15}\text{N}$ in Hobsons Bay ($\delta^{15}\text{N}$ of *Botryocladia* ~ 12.0) were substantially higher than the $\delta^{15}\text{N}$ of the high volume of NO_x discharged to this region by the Yarra River ($\delta^{15}\text{N} = 8.0$, Table 2), and higher than $\delta^{15}\text{N}$ values of *Capreolia* at Intertidal sites 21 ($\delta^{15}\text{N} = 9.0$) and 22 ($\delta^{15}\text{N} = 7.5$), which are more influenced by the Yarra River plume, which follows the eastern shoreline. The elevated $\delta^{15}\text{N}$ values in Hobsons Bay may be due to infrequent excursions of WTP-sourced N, but could also be influenced by fractionation in the sediments. Hobsons Bay has characteristics typical of other areas with ε_{sed} greater than zero, including low denitrification efficiency (28%, Berelson *et al.* 1998), high organic load and poorly oxygenated surface sediments (Lehmann *et al.* 2004; Alkhatib *et al.* 2012).

Values of $\delta^{13}\text{C}$ of *Botryocladia* deployed in the bay showed broadly similar, but more complex, spatial patterns than those of $\delta^{15}\text{N}$. Plants discriminate against $^{13}\text{CO}_2$ during photosynthesis, but the degree of discrimination varies among species using different photosynthetic pathways (e.g. C_3 , C_4 and CAM plants). Macroalgae with $\delta^{13}\text{C}$ smaller than -30‰ (such as

Botryocladia) typically occur in subtidal or shaded intertidal habitats where they are often light rather than carbon limited, and rely on CO_2 diffusion for carbon uptake, a process that actively discriminates against ^{13}C (Marconi *et al.* 2011). Proximity to the WTP was expected to reduce the likelihood of nutrient limitation and increase the likelihood of carbon limitation, which in turn will decrease the discrimination against ^{13}C , and so increase $\delta^{13}\text{C}$. The values of $\delta^{13}\text{C}$ of *Botryocladia* offshore from the north-eastern end of the Bellarine Peninsula were inconsistent with the broad pattern shown by $\delta^{15}\text{N}$ and with proximity to the WTP. These $\delta^{13}\text{C}$ outliers probably resulted from interactions among carbon, nutrient and light limitation. Light available to *Botryocladia* was probably affected by shading from nutrient-dependent epiphytes, and those along the shipping channel by turbid plumes from passing ships. The complexity of the $\delta^{13}\text{C}$ pattern is consistent with previous studies that have shown $\delta^{15}\text{N}$ to provide a more reliable estimate of nutrient/sewage influence than does $\delta^{13}\text{C}$ (Roelke and Cifuentes 1997; Gaston *et al.* 2004; Dudley *et al.* 2010).

Aquatic macrophytes with $\delta^{13}\text{C}$ between -10 and -30‰ (such as *Capreolia* and *Hormosira*) typically inhabit intertidal zones and use HCO_3^- as an inorganic carbon source, and usually have a carbon-concentrating mechanism (Marconi *et al.* 2011). Whereas $\delta^{15}\text{N}$ values for intertidal algae and filter-feeders were all spatially correlated, some of the spatial variation in $\delta^{13}\text{C}$ in *Capreolia* (e.g. high values at Sites 4, 22 and 23, Fig. 3) appeared unrelated to the likely exposure to the WTP plume. However, the *Capreolia* nearest to the WTP, at Point Cook (Site 17, Fig. 3), had the highest $\delta^{13}\text{C}$ value and this was similar to the high $\delta^{13}\text{C}$ values measured adjacent to another treated-sewage discharge (Parry 2021).

Regions of low ^{15}N , N-fixation by seagrass

Nitrogen fixation by sulfate-reducing bacteria associated with seagrass roots is a major source of light N ($\delta^{15}\text{N}$ of ~ 0) and

often provides for a significant proportion of the nitrogen needs of seagrasses (Welsh 2000), including *Zostera muelleri* (Udy and Dennison 1997). In this study, the lowest $\delta^{15}\text{N}$ signatures of *Botryocladia* and *Mytilus* were recorded in Corio Bay and Swan Bay, where near-zero $\delta^{15}\text{N}$ signatures were also recorded for seagrasses (Cook *et al.* 2015; Jenkins *et al.* 2015; Hirst *et al.* 2016; Russell *et al.* 2018). There have been several interpretations of the low values of $\delta^{15}\text{N}$ of seagrass at particular sites in Port Phillip Bay. Cook *et al.* (2015) suggested that the small difference between $\delta^{15}\text{N}$ of *Ulva* and seagrass (*Zostera muelleri*) at the same sites in Port Phillip Bay was due to isotopic fractionation in the sediment rather than N-fixation, and their direct measurements suggested that N-fixation was only a minor ($\sim 15\%$) source of N in seagrass. Hirst *et al.* (2016) suggested that low $\delta^{15}\text{N}$ of seagrass in Swan Bay may be due to either N-fixation or recycling within the sediment. Subsequently, Russell *et al.* (2018) found that $\delta^{15}\text{N}$ of pore water was linearly related to that of seagrass, and where $\delta^{15}\text{N}$ of seagrass was near zero (Corio Bay and Swan Bay), so was $\delta^{15}\text{N}$ of NH_4^+ in pore water.

The low $\delta^{15}\text{N}$ in both pore water and the water column at Corio Bay and Swan Bay suggests that the ultimate source of this nitrogen is nitrogen-fixing bacteria. The enclosed geography of these bays looks to be important in the retention of this nitrogen. Both bays have small entrances that reduce water exchange and ensure minimal export of local seagrass production. Seagrass leaves shed by storms or when rhizomes are consumed by black swans become detritus and are buried and broken down by microbes, releasing their N to pore water, which is then recycled by plants. Both bays also have limited fetch, which helps retain fine sediments, which in turn retain more NH_4^+ in pore water (Hirst and Jenkins 2017). The low export of seagrass production contrasts with sites on the east of Port Phillip Bay (including Blairgowrie, Rye and South Sands, Hirst and Jenkins 2017) where most seagrass production is exported, sediment is coarser with less retained organic matter, NH_4^+ concentrations in pore water are an order of magnitude lower and, in contrast to Swan Bay, the addition of nutrients to the sediment increases the rate of growth of seagrass (Hirst and Jenkins 2017).

The lower $\delta^{15}\text{N}$ values found for all species in the Geelong Arm (Sites 5–12, Fig. 3a) than those on the east of Port Phillip Bay (Sites 20–27, Fig. 3a) are likely to be in part the result of seagrass contributing more light N in this region. Biota at some sites along the northern shoreline of the Geelong Arm also had unexpectedly low $\delta^{15}\text{N}$ values, suggesting that this area also has a source of light nitrogen as well as being influenced by the plume of the WTP. The $\delta^{15}\text{N}$ value was low at Sites 10 and 12, but higher at Site 11. The lower $\delta^{15}\text{N}$ values that occur at some sites in the Geelong Arm may result from their proximity to geographic features that trap large amounts of seagrass wrack that release light ^{14}N . Similar fine-scale variation in $\delta^{15}\text{N}$ of *in situ* macroalgae was found to be due to decomposition of organic matter from seasonal seaweed standings (Lemesle *et al.* 2016).

The exceptionally low $\delta^{15}\text{N}$ values found in all species suspended in the water column in seagrass habitats indicates that some of the N fixed by seagrass is exported to the water column. This will be greater where extensive meadows of seagrass occur (Corio Bay, Swan Bay, and Point Wilson quarry) and probably where seagrass drift accumulates because of the groyne-like geographic features. The amount of N exported to the water column as a result of nitrogen fixation from seagrass is difficult to estimate from available data, but an approximate estimate was obtained for Swan Bay, using best estimates of $\delta^{15}\text{N}$ for water outside and within Swan Bay. The value of $\delta^{15}\text{N}$ of water outside Swan Bay was estimated from its value at the entrance to Port Phillip Bay, estimated from the mean $\delta^{15}\text{N}$ of *Capreolia* and *Hormosira* at Point Lonsdale [4.8 ± 0.3 ($N = 8$)]. The value of $\delta^{15}\text{N}$ of water in Swan Bay was estimated from $\delta^{15}\text{N}$ of *in situ* *Mytilus* in Swan Bay [7.5 ± 0.4 ($N = 2$) – 3.5 ± 0.3 (trophic fractionation) = 4.0 ± 0.7]. These values suggest that, if $\delta^{15}\text{N}$ of N fixed by seagrass is 0, then the proportion of nitrogen in the water column fixed by seagrass is $(4.8 - 4.0)/4.0 = 0.20$, but the error range includes zero. More measurements are clearly needed, particularly of $\delta^{15}\text{N}$ of *in situ* macroalgae that source their N from the water column.

Changes following transplantation

The rate of change of $\delta^{15}\text{N}$, when an organism is introduced into an environment with a distinctively different ^{15}N signal, depends on tissue growth and N turnover associated with tissue maintenance. The contribution of these two processes will vary, depending on the rate of growth (Fry and Arnold 1982; Gaye-Siessegger *et al.* 2004; McIntyre and Flecker 2006; Ankjærø *et al.* 2012). The rate of change of $\delta^{15}\text{N}$ will be slowest when there is no growth and all change is due to tissue turnover for maintenance. Where there is new growth, the newly incorporated N will increase with growth rate, because new tissue would be expected to carry primarily the ^{15}N signature of the new environment.

The rapid increase in $\delta^{15}\text{N}$ of *Capreolia* and *Hormosira* in the first week, and of nitrogen content in the first 3 weeks, were probably due to 'luxury uptake', where nutrients are placed in intracellular storage rather than used immediately for protein growth (Kilham and Hecky 1988). Subsequent increases in $\delta^{15}\text{N}$ occurred more slowly because they were due to algal growth. The rate of change of $\delta^{15}\text{N}$ in transplanted *Galeolaria* anomalously reached an asymptote well below the $\delta^{15}\text{N}$ of *in situ* *Galeolaria*, probably because they were placed in a non-optimal micro-environment where growth slowed.

There are few measurements of tissue-stable isotope turnover rates (Dattagupta *et al.* 2004; McIntyre and Flecker 2006), although primary producers have higher variance in $\delta^{15}\text{N}$ than do secondary consumers (Cabana and Rasmussen 1996; Post 2002; McIntyre and Flecker 2006). Aguiar *et al.* (2003) suggested that the nitrogen pool in the macroalgae *Ulva* turns over completely every 12–15 days. This estimate of turnover of nitrogen is much faster than those of macroalgae

from this study (6–12 weeks and 10 weeks), probably for two reasons. First, *Ulva* is widely recognised as a species associated with areas of high nutrients and has a high rate of growth. Second, the estimate for *Ulva* by Aguiar *et al.* (2003) was an extrapolation of the rate over the first 8 days, when most nitrogen probably went to storage ('luxury uptake') rather than growth. Estimated turnover rates of nitrogen in *Galeolaria* and *Mytilus* ($T_{1/2}$ = 90 days and 234 days respectively) appear similar to the limited data available for other species. McIntyre and Flecker (2006) reviewed available estimates of turnover rates and found half-lives ($T_{1/2}$) between 156 and 784 days for krill, 69 days for *Elimia* (snail), 99–231 days for *Littorina* (snail), and very variable rates for fish (3 to >173 days), depending largely on their size and growth rate. Subsequent estimates of $T_{1/2}$ for nitrogen in fast-growing Atlantic cod, in heart and white muscle, were 31–78 days (Ankjærø *et al.* 2012). However, Dubois *et al.* (2007) found much more rapid turnover rates, $T_{1/2}$ = 14 days for Pacific oysters and $T_{1/2}$ = 15 days for *Mytilus*. These latter estimates are higher than estimates for algae, and appear unrealistically high, especially as mussels only grew 38% over 90 days.

Preferred species for ongoing monitoring

Values of $\delta^{15}\text{N}$ in all species showed similar spatial patterns around Port Phillip Bay, suggesting that all are satisfactory for monitoring sources of N with distinctive $\delta^{15}\text{N}$ values. But the absence of intertidal reef habitat adjacent to the WTP limited the usefulness of species dependent on this habitat, especially *Capreolia* and *Hormosira*. Both *Galeolaria* and *Mytilus* can also establish on small coastal man-made structures. The absence of *Hormosira* on the intertidal reef at Point Cook is consistent with its low tolerance of high DIN (Parry 2021), which suggests that it is the least suitable species for monitoring $\delta^{15}\text{N}$ of WTP-sourced N.

The suitability of different species depends on the time scale of the questions being posed, and the costs of sampling and analysis. Species and tissues with high turnover rates (e.g. algal tissues) are more responsive to short-term changes in the discharge of ^{15}N , whereas slower-growing taxa (e.g. *Galeolaria* and *Mytilus*) and tissues with longer turnover rates (e.g. muscle, Lorrain *et al.* 2002) are better suited to providing a time-integrated measure of the area of influence of discharged ^{15}N (Gartner *et al.* 2002; Fertig *et al.* 2009; Raimonet *et al.* 2013). The costs of collection and analysis are also important. Collection costs are lower for intertidal species than for those deployed on moorings. But the mean time taken to dissect and grind samples was generally greater for intertidal species (*Capreolia* 20–25 min, *Hormosira* 5 min, *Galeolaria* 35 min) than for those deployed on moorings (*Botryocladia* 5 min, *Mytilus* 14 min). Species attached to offshore moorings provide the only means of measuring $\delta^{15}\text{N}$ in non-coastal regions and are also better suited to monitoring long-term trends from sources with a large footprint such as the WTP, because,

unlike intertidal species, they are not affected by small local inputs.

Of the five species considered in this study, *Mytilus* appears the best species for monitoring long-term change in ^{15}N , and for ground-truthing nutrient models. The current study deployed large mussels, but these are not ideal for detecting temporal trends or for ground-truthing models because $\delta^{15}\text{N}$ measured at the end of their deployment is affected by their initial $\delta^{15}\text{N}$. Correcting for the influence of the latter requires a complex model of *Mytilus* growth. However, if small fast-growing *Mytilus* were deployed, essentially all the ^{15}N in the *Mytilus* then results from new growth during the deployment period, so no correction for the starting ^{15}N signal is required. Post-larval shrimp, which had a four-fold increase in weight, showed essentially constant isotopic values, reflecting their new diets (Fry and Arnold 1982). Similarly, Trueman *et al.* (2005) found that following a diet switch in Atlantic salmon, the $\delta^{15}\text{N}$ of muscle and liver reached equilibrium with their new diet after a three-fold increase in body mass, equivalent to 8 months of growth under laboratory conditions. Commercial hatcheries in Port Phillip Bay currently produce 1 mm length mussels suitable for deployment, and when deployed on mussel farms they grow to ~5–10 mm over 2 months, when their $\delta^{15}\text{N}$ should just reflect their exposure to ^{15}N over the period of their deployment.

Supplementary material

Supplementary material is available [online](#).

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Data availability. The data that support this study will be shared upon reasonable request to the corresponding author.

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