

# Nitrogen stable isotope values of large-bodied consumers reflect urbanization of coastal catchments

F. Y. Warry<sup>1,2,\*</sup>, Paul Reich<sup>1,2</sup>, Ryan J. Woodland<sup>1,4</sup>, James R. Thomson<sup>2</sup>,  
Ralph Mac Nally<sup>3</sup>, Perran L. M. Cook<sup>1</sup>

<sup>1</sup>Water Studies Centre, School of Chemistry, Monash University, Clayton, VIC 3800, Australia

<sup>2</sup>Department of Environment, Land Water and Planning, Arthur Rylah Institute for Environmental Research, Heidelberg, VIC 3084, Australia

<sup>3</sup>Institute for Applied Ecology, University of Canberra, Bruce, ACT 2617, Australia

<sup>4</sup>Present address: Chesapeake Biological Laboratory, University of Maryland Center for Environmental Science, PO Box 38, Solomons, MD 20688, USA

**ABSTRACT:** Anthropogenic nitrogen inputs to aquatic ecosystems can detrimentally affect ecosystem function; therefore, we need methods that identify nitrogen sources consistently among catchments. Nitrogen sources may be distinguished with stable isotope values ( $\delta^{15}\text{N}$ ). This study tested if the isotopic values of biota and inorganic oxides of nitrogen ( $\text{NO}_3^- + \text{NO}_2^-$ , hereafter  $\text{NO}_x^-$ ) within estuaries were predictably influenced by anthropogenic land use in catchments distributed across a coastline. The  $\delta^{15}\text{N}$  values of  $\text{NO}_x^-$ , autotrophs and consumers (fish) were measured. Increased catchment urbanization was reflected in higher  $\delta^{15}\text{N}$  values of larger-bodied fish consumers but not smaller-bodied consumers, autotrophs or  $\text{NO}_x^-$ , which may reflect the time-averaging effect of longer tissue equilibration times in the larger fish. The proportion of the catchment subject to intensive agriculture did not relate to isotopic values of estuarine biota or  $\text{NO}_x^-$ . The lack of relationships between intensive agriculture and  $\delta^{15}\text{N}$  in the estuaries studied may result from the mixing of nitrogen sources within the catchments studied, non-uniformity in biogeochemical transformations of nitrogen during transport from agricultural sources to estuaries, or insufficiencies of broad land-use data for describing impacts of agriculture on nitrogen isotopic pools. The  $\delta^{15}\text{N}$  values of consumers with slow tissue turnover appear most useful for identifying the incorporation of urban nutrient inputs into estuarine food webs at catchment scales.

**KEY WORDS:** Estuary · Watershed · Non-point source · Land–water coupling · Nitrate · Fish · Seagrass · *Zostera* · *Phragmites australis*

— Resale or republication not permitted without written consent of the publisher —

## INTRODUCTION

Anthropogenic land-use change has dramatically increased the rate at which bioavailable nitrogen enters aquatic ecosystems (Galloway et al. 2003). Agricultural, industrial and urban development increase nitrogen inputs, which can threaten aquatic ecosystems by disrupting ecological processes including primary production, nutrient cycling, energy transfer

and faunal population dynamics. Sources of anthropogenic nitrogen to aquatic ecosystems are often uncertain due to mixed catchment land uses and biogeochemical transformations (Kaushal et al. 2011). As such, methods that can identify the incorporation of anthropogenic nitrogen into aquatic food webs are valuable for ecosystem researchers and managers.

Stable isotopes of nitrogen (expressed as  $\delta^{15}\text{N}$  in ‰) have been used to identify sources of anthropogenic

nitrogen in aquatic ecosystems. Nitrogen from wastewater and manure has high  $\delta^{15}\text{N}$  values relative to other sources (McClelland & Valiela 1998), whereas nitrogen from inorganic fertilizers has low  $\delta^{15}\text{N}$  values because it is generated from isotopically light atmospheric nitrogen through the Haber-Bosch process (Erisman et al. 2008). However, biogeochemical processes, including denitrification and volatilization, can transform the isotopic composition of nitrogen within, or during transport to, aquatic ecosystems (Wankel et al. 2009). Nitrogen derived from inorganic fertilizers (i.e. with low  $\delta^{15}\text{N}$  values) can become enriched before entering downstream water bodies by undergoing denitrification (Anderson & Cabana 2005, Diebel & Vander Zanden 2009), which discriminates against the heavier isotope (Burns et al. 2009).

Nitrogen isotopes have been widely used to detect nitrogen from point sources of treated wastewater entering freshwater (Hood et al. 2014), estuarine (Pitt et al. 2009) and marine (Costanzo et al. 2005, Connolly et al. 2013) ecosystems. Relationships between non-point sources of anthropogenic nitrogen, e.g. urban and agricultural land uses, have been increasingly investigated and detected in freshwater stream networks (Anderson & Cabana 2005) and lake systems (Vander Zanden et al. 2005). Agricultural (Anderson & Cabana 2005, Diebel & Vander Zanden 2009, Barnes & Raymond 2010) and urban (Kaushal et al. 2011, Barnes & Raymond 2010) land uses within catchments have affected  $\delta^{15}\text{N}$  values in freshwater ecosystems and these relationships have been observed at catchment scales spanning hundreds of kilometres (e.g. Anderson & Cabana 2005). Variation in  $\delta^{15}\text{N}$  among estuaries has also been linked to urbanization (Bannon & Roman 2008, Olsen et al. 2010) and human population density (Cole et al. 2006, Bannon & Roman 2008). However, these investigations were done at catchment- or system-specific spatial scales spanning tens of kilometres. Far fewer studies have assessed relationships between catchment land use and  $\delta^{15}\text{N}$  in estuaries at catchment scales spanning hundreds of kilometres, and results have been equivocal from those studies. Christiaen et al. (2014) reported no relationship between the degree of catchment population density and seagrass foliar  $\delta^{15}\text{N}$  on a global scale. Valiela et al. (2014) found that within-estuary transformations obscured effects of land use on the  $\delta^{15}\text{N}$  values of suspended particulate organic matter (SPOM), which were observed in lower salinity reaches of estuaries distributed over hundreds of kilometres. However, Cole et al. (2004) observed positive relationships between

urbanization and macroalgae  $\delta^{15}\text{N}$  at continental scales. Hoffman et al. (2012) also observed positive relationships between human population density and the  $\delta^{15}\text{N}$  of fish larvae in riverine estuaries at catchment scales, but these relationships were modulated by the degree of hydrologic flushing.

Estuaries are the termini of stream networks and act as receiving bodies for upstream inputs; they could be expected to have an integrated isotope pool reflective of catchment land uses and marine influences. Catchments distributed over hundreds of kilometres often have multiple land uses and variation in factors influencing nutrient transport and biogeochemical transformation, such as hydrology (i.e. groundwater inputs) and geology (e.g. soil type). Estuaries are also sites of intense biogeochemical transformations (Valiela et al. 2014) and potentially dynamic isotopic pools owing to the interplay between marine and freshwater inputs. Tracing anthropogenic nitrogen into aquatic food webs with stable isotopes requires nitrogen sources with distinct isotopic signatures and known transformation of those isotopic signatures from their origin to the aquatic ecosystem. The potential for mixing of nitrogen sources and non-uniformity in biogeochemical transformations during nutrient transport is greater when the spatial scale of investigation increases. Identifying whether  $\delta^{15}\text{N}$  values of estuarine inorganic nitrogen or biota can provide evidence of anthropogenic nutrient uptake predictably among catchments would inform environmental condition assessments and demonstrate coupling between terrestrial catchments and estuaries. If there are consistent relationships, then  $\delta^{15}\text{N}$  values may be valuable indicators of non-point source nutrient uptake for coast-wide monitoring.

Here, nitrogen isotope values of estuarine  $\text{NO}_3^- + \text{NO}_2^-$  (hereafter  $\text{NO}_x^-$ ), autotrophs and consumers were used to explore whether variation in  $\delta^{15}\text{N}$  was related to broad patterns of catchment land use. Our hypotheses are based on the expectation that different proportions of anthropogenic land use within catchments will exert different influences on the isotopic pool in estuaries. Increased human wastewater inputs, from diffuse urban or point sources, might increase the proportion of isotopically heavy nitrogen and enrich  $\delta^{15}\text{N}$  values in estuaries. An increased proportion of the catchment subject to intensive agriculture may also increase the proportion of isotopically heavy nitrogen comprising estuarine nitrogen pools, compared to catchments with greater proportions of natural vegetation. Intensive agriculture involves the production of animal manure and application of or-

ganic and inorganic fertilizers. Although inorganic fertilizers are originally isotopically light, these nutrients are typically fractionated by biogeochemical processes, becoming more isotopically heavy, before entering waterways (Wankel et al. 2009). Marine or tidal exchange may mediate inputs from agricultural and urban land uses (Hoffman et al. 2012), with  $\delta^{15}\text{N}$  values of estuarine  $\text{NO}_x^-$ , autotrophs and consumers approaching those of marine signatures in highly flushed systems. These expectations may not apply to all taxa, particularly mobile consumers with plastic feeding behaviours or plants capable of assimilating nitrogen via multiple pathways.

Based on these *a priori* expectations, we tested whether (1) urban and agricultural land use within catchments could explain patterns of  $\delta^{15}\text{N}$  in estuarine  $\text{NO}_x^-$ , autotrophs and fish among catchments; and (2) relationships between catchment land use and  $\delta^{15}\text{N}$  were consistent among multiple trophic levels.

## MATERIALS AND METHODS

### Study locations and survey design

This study was undertaken in the estuarine reaches of 31 rivers throughout Victoria, southeastern Australia, within temperate latitudes  $38.86$  to  $37.57^\circ\text{S}$  (Fig. 1, Table S1 in Supplement 1 at [www.int-res.com/articles/suppl/m542p025\\_supp.pdf](http://www.int-res.com/articles/suppl/m542p025_supp.pdf)) from 2010 to 2012. Estuaries were selected across a gradient of catchment land-use intensity. The estuaries included some permanently open to the sea and some with entrances that were open intermittently. The estuaries were representative of Victorian daily tidal amplitudes ( $\sim 0.3$  to  $2.8$  m; obtained from the Australian Bureau of Meteorology at [www.bom.gov.au/](http://www.bom.gov.au/) [accessed September 2014]) and catchment land uses (Stein et al. 2014) (Table S1 in Supplement 1).

A total of 11 estuaries within Port Phillip and Western Port were sampled in 2010, 2011 and 2012. A total

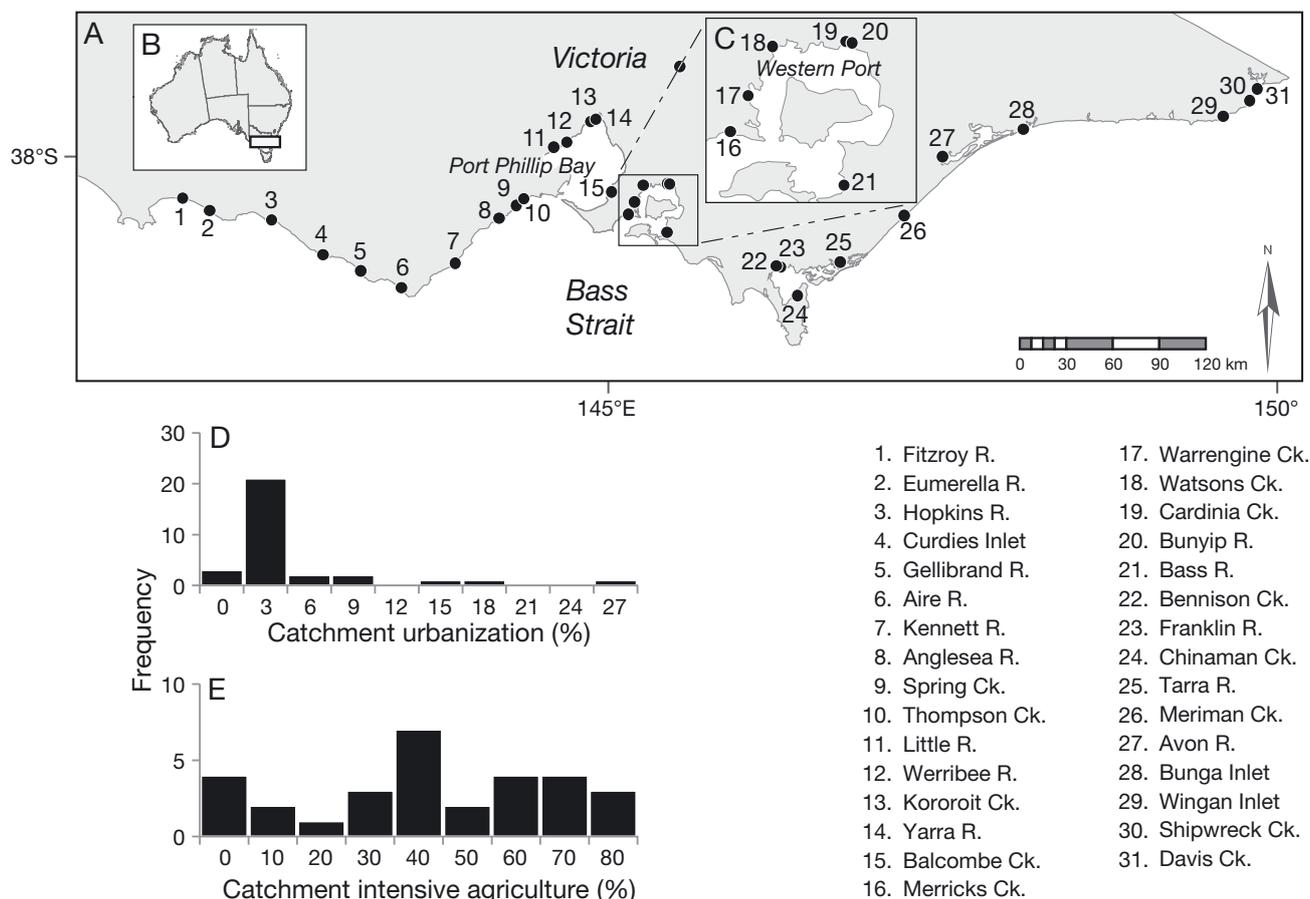


Fig. 1. (A) Location of riverine estuaries within Victoria sampled during this study, (B) location of Victoria (overlaid with outline rectangle of panel A) within Australia, (C) enlarged map of estuaries sampled in Western Port, (D) frequency distributions of catchment urbanization and (E) intensive agriculture as percentage of catchment area for the catchments of estuaries studied.

R.: River; Ck.: Creek

of 10 estuaries were sampled in western Victoria during 2011, and 10 in eastern Victoria during 2012. Estuaries were sampled in the east and west of the state in different years due to logistical constraints, and this was addressed in statistical analyses. However, estuaries in both the east and west of the state were representative of the gradient of daily tidal amplitude and of land use in both regions. Sampling was undertaken in the austral late summer and autumn (February and March, hereafter 'autumn') and spring (October and November) during each year for each estuary.

### Sampling

Fish and autotrophs were collected for analysis of stable isotope ratios of nitrogen ( $^{15}\text{N}/^{14}\text{N}$ ). Multiple individuals ( $n \geq 5$ ) of each fish species were collected using single-wing fyke nets, multi-panel mesh nets and beach seine nets (see Warry et al. 2013 for net specifications and deployment). Only adult life-stages were used. Individuals of 4 species with varying body size and trophic level were retained when caught. These species also occurred consistently among the estuaries surveyed. Black bream *Acanthopagrus butcheri* can reach 500 mm in length, 29 yr old and is an opportunistic invertivore feeding at trophic level  $3.5 \pm 0.5$ . Yelloweye mullet *Aldrichetta forsteri* can reach 500 mm in length, 7 yr old and is a detritivore, feeding at trophic level  $2.5 \pm 0.3$ . Flathead gudgeon *Philypnodon grandiceps* can reach 120 mm in length and is an opportunistic invertivore that can tend towards piscivorous, feeding at trophic level  $3.8 \pm 0.6$ . Eastern bluespot goby *Pseudogobius* sp. 9 can reach 60 mm in length and is a zoobenthivore, feeding at trophic level  $2.9 \pm 0.4$ . These trophic levels (means  $\pm$  SD) for all species were obtained from FishBase and based on previously conducted diet studies (www.fishbase.org). Flathead gudgeon *P. grandiceps* were sampled from polyhaline to mesohaline zones. Other species were sampled from euhaline to polyhaline zones.

Replicate samples ( $n = 3$ ) of each autotroph were collected from each estuary where they occurred. Each replicate was taken from a separate plant. Samples of the common reed *Phragmites australis* and seagrass *Zostera nigricaulis* and *Z. muelleri* were collected by removing several green leaves. Samples of green filamentous algae were collected by detaching algae from the benthos. Green filamentous algae and seagrass were sampled from euhaline to polyhaline zones. *P. australis* was sampled from mesohaline zones.

Water samples for analyses of  $\delta^{15}\text{N}$  and  $\delta^{18}\text{O}$  of  $\text{NO}_x^-$  (i.e. nitrates and nitrites;  $\delta^{15}\text{N}\text{-NO}_x^-$  and  $\delta^{18}\text{O}\text{-NO}_x^-$ ) were collected from the surface at the oligohaline section of each estuary. Water was filtered using  $0.45 \mu\text{m}$  ashed GF/F glass fibre filters and 100 ml of filtrate was retained for analyses. One sample was taken from each estuary on each sampling occasion.

Fish, autotroph and filtered water samples were stored on ice during transportation to the laboratory, where they were frozen and stored at  $-18^\circ\text{C}$ .

### Sample preparation and analysis

White muscle tissue, immediately ventral to the anterior region of the dorsal fin, was used for isotope analysis of fish because this tissue exhibits less isotopic variability than others (Pinnegar & Polunin 1999) and integrates dietary components over several months, depending on growth rates (Hesslein et al. 1993). The most recent growth of leaves or fronds of autotrophs were prepared for isotope analyses. Epibionts were removed from leaves and fronds. Samples were washed in distilled water, dried to constant weight (48 h at  $60^\circ\text{C}$ ) and ground to a fine, homogenous powder.

Nitrogen stable isotope ratios from samples collected in 2010 and autumn 2011 were analysed at Natural Isotopes, Edith Cowan University, Western Australia, on a calibrated ANCA-GSL elemental analyser interfaced to a 20-20 continuous-flow isotope ratio mass-spectrometer (Europa). Nitrogen stable isotope ratios from samples collected in 2012 and spring 2011 were analysed at the Water Studies Centre, Monash University, Victoria, Australia, on a calibrated ANCA-GSL2 elemental analyser interfaced to a Hydra 20-22 continuous-flow isotope ratio mass-spectrometer (Sercon). At both laboratories, reference materials of known elemental composition and isotopic ratios that bracketed sample values were interspaced with the samples for calibration. Reference materials at different weights were used to correct for instrument linearity. Frozen water samples were analysed for  $\delta^{15}\text{N}\text{-NO}_x^-$  and  $\delta^{18}\text{O}\text{-NO}_x^-$  using the bacterial denitrifier method (Sigman et al. 2001, Casciotti et al. 2002) at the Colorado Plateau Stable Isotope Laboratory, Arizona, USA. Stable isotope data were expressed in the delta notation ( $\delta^{13}\text{C}$ ,  $\delta^{15}\text{N}$  and  $\delta^{18}\text{O}$ ; Fry 2006) relative to the stable isotopic ratio of Vienna Pee Dee Belemnite (VPDB) for carbon, air for nitrogen and Vienna Standard Mean Ocean Water (VSMOW) for oxygen.

## Response variables and predictors

Two estuaries receive direct inputs from a wastewater treatment plant, the Western Treatment Plant at Werribee that treats ~50 % of Melbourne's sewage and discharges up to ~90 000 Ml yr<sup>-1</sup> of tertiary treated effluent. Little River is within the mixing zone of the Western Treatment Plant stipulated on Victorian Environmental Protection Agency (EPA) discharge licences. The Werribee River is just outside the official mixing zone; however, agriculture on the floodplain immediately adjacent to the estuary receives recycled water from the Western Treatment Plant for irrigation. The Werribee River estuary is also immediately adjacent to the treatment plant and previous work indicates that groundwater inputs to the estuary are partly derived from the treatment plant (Wong et al. 2014, 2015). These sites were considered subject to point source delivery of anthropogenic nitrogen directly to the estuary that would override any effects of catchment land use. As such, these sites were not included in modelling of relationships between catchment land use and  $\delta^{15}\text{N}$ . No other estuaries studied are directly subject to treated wastewater discharge.

The Fitzroy, Hopkins, Curdies, Yarra and Bunyip river estuaries are within catchments where tertiary treated wastewater is discharged to surface waters in freshwater reaches of the river network (Geoscience Australia, National Wastewater Treatment Plant Database [http://www.ga.gov.au/metadata-gateway/metadata/record/gcat\\_74625](http://www.ga.gov.au/metadata-gateway/metadata/record/gcat_74625) [accessed August 2015]). These wastewater discharges were not expected to have substantial influence on the isotopic composition of estuarine nitrogen pools as they represented a maximum of 11 % of the annual total nitrogen load entering the Yarra River estuary and a maximum of 6 % for the other estuaries. These loads were estimated using daily flow measurements, nutrient monitoring data (<http://data.water.vic.gov.au/monitoring.htm> [accessed December 2014]) and wastewater treatment plant discharge limits stipulated on Victorian EPA discharge licences ([www.epa.vic.gov.au/](http://www.epa.vic.gov.au/) [accessed August 2015]; see Supplement 2 at [www.int-res.com/articles/suppl/m542p025\\_supp.pdf](http://www.int-res.com/articles/suppl/m542p025_supp.pdf)). Therefore, these estuaries were included in modelling of relationships between catchment land use and  $\delta^{15}\text{N}$ . Data for the catchment urbanization and catchment intensive agriculture predictors were obtained from the National Environmental Stream Attributes database v.1.1 (Stein et al. 2014) and Bureau of Rural Sciences' 2005/06 Land Use of Australia Version 4 maps ([www.agriculture.gov.au/abares/aclump](http://www.agriculture.gov.au/abares/aclump)) (accessed

December 2014). The catchment urbanization predictor variable describes the percentage of the catchment subject to residential, industrial or urban commercial development. It was included in analyses to represent integrated diffuse sources of human-derived domestic/industrial wastewater, e.g. leaking sewage infrastructure, storm water, nutrient application to residential gardens and accelerated nutrient delivery via impervious surfaces. The catchment intensive agriculture variable describes the percentage of the catchment subject to intensive animal production, intensive plant production (horticulture and irrigated cropping) and grazing of modified pastures. It was included in analyses to represent integrated diffuse sources of nutrients derived from intense agriculture including animal manure and inorganic fertilizers. The aggregation of agricultural land uses employing inorganic and organic fertilizers was considered appropriate because isotopically light inorganic fertilizers (Kaushal et al. 2011) are typically fractionated by biogeochemical processes, becoming isotopically heavy, before they enter water-ways (Anderson & Cabana 2005, Wankel et al. 2009). Analyses were also performed with intensive animal production, intensive plant production and grazing of modified pasture land uses treated as individual predictor variables. This had no effect on modelling outcomes (see Supplement 3 at [www.int-res.com/articles/suppl/m542p025\\_supp.pdf](http://www.int-res.com/articles/suppl/m542p025_supp.pdf)), so only analyses using the aggregate intensive agriculture predictor variable are presented.

The tidal exchange variable approximated the maximum percentage of estuary volume that would be exchanged with the ocean over a daily tidal cycle and was used to characterize the extent of tidal flushing that may mediate an influence of wastewater or fertilizer inputs to the estuary. Tidal exchange was derived from estuary volume, mean daily tidal amplitude and a measure of mouth geomorphology. Estuary volumes were calculated using areas derived from aerial photography and depth measurements taken during sampling activities. Mean daily tidal amplitudes were calculated from the previous 10 yr of measurements at intervals of 6 min from the nearest tide gauge. Tidal volume of the estuary was divided by the estuary volume and multiplied by a factor of mouth geomorphology (i.e. 0 if the estuary was closed; 1 if the estuary was completely open) to provide a measure of potential tidal exchange used in the mixed models.

The response variables were the  $\delta^{15}\text{N}$  values of inorganic oxides of nitrogen ( $\text{NO}_x^-$ ), *Phragmites australis*, seagrass *Zostera* spp., green filamentous algae, black bream *A. butcheri*, yelloweye mullet *A.*

*forsteri*, flathead gudgeon *P. grandiceps* and eastern bluespot goby *Pseudogobius* sp. 9. Means of samples for each response taken from each estuary on each sampling occasion were used. Response data were ln-transformed (following the addition of a constant to remove negative values) to meet distributional assumptions.

### Analyses

Relationships between land use within catchments and  $\delta^{15}\text{N}$  values of estuarine  $\text{NO}_x^-$ , autotrophs and fish were assessed using linear mixed models. Estuary and year were included as random effects in all models. Catchment urbanization, catchment intensive agriculture and tidal exchange were included as continuous predictors. Season (autumn or spring) was included as a categorical variable. Interactions between season and land use predictors, tide and land use predictors, and among land use predictors were included in the suite of models assessed (Table S5 in Supplement 4 at [www.int-res.com/articles/suppl/m542p025\\_supp.pdf](http://www.int-res.com/articles/suppl/m542p025_supp.pdf)).

Model selection was done using Akaike's information criterion corrected for small sample size ( $\text{AIC}_c$ ; Symonds & Moussalli 2011).  $\text{AIC}_c$  values were converted to AIC weights ( $\text{AIC}_w$ ) to provide normalized relative model likelihoods.  $\text{AIC}_w$  represents the probability that a model is the best model given the data and the suite of candidate models assessed.  $\text{AIC}_w$  ranges from 0 to 1. An  $\text{AIC}_w$  of 0 indicates zero probability that a given model is the best.  $\text{AIC}_w$  values approaching 1 indicate high probability that a given model is the best (Wagenmakers & Farrell 2004).

The performance of the best (highest  $\text{AIC}_w$ ) models was assessed using 10-fold cross-validation (Wang et al. 2014). The data set was randomly divided into 10 mutually exclusive subsets (or folds) of equal size. Each of the 10 subsets was used once to test models trained on the remaining 9 subsets. The cross-validated  $R^2$  ( $R^2_{cv}$ ), defined as the mean coefficient of determination over the 10 validation tests, describes a model's expected predictive capacity: the ability of the model to estimate the response in cases independent of the training data. Good predictive performance is indicated by  $R^2_{cv} > 0.5$  (Triba et al. 2015). Analyses were done in R (R Core Team 2014).

The relative importance of predictors in explaining patterns in responses was calculated by summing the  $\text{AIC}_w$  values to provide variable probabilities, from which odds ratios were calculated to adjust for unequal prior probabilities of variables. Different pre-

dictors appeared with unequal frequency in the suite of candidate models, potentially biasing summed  $\text{AIC}_w$  values towards variables with higher prior probabilities (those included in more candidate models). We used odds ratios to correct this bias: odds ratio = posterior odds/prior odds, where prior odds = prior probability/(1 - prior probability), posterior odds = posterior probability/(1 - posterior probability). Prior probabilities were calculated as the number of models in which a particular predictor variable occurred divided by the total number of models assessed. Posterior probabilities for a particular predictor variable were the summed  $\text{AIC}_w$  values of all models in which that predictor occurred. Adjusted variable weights ( $W_{\text{var}}$ ) were calculated as  $W_{\text{var}} = \text{odds ratio}/(1 + \text{odds ratio})$  and are equivalent to the probability that a given predictor variable is included in the best model variant, assuming all variables have equal prior probability (prior odds = 1).  $W_{\text{var}}$  ranges from 0 to 1, with values  $> 0.8$  indicating strong evidence that a variable is a predictor for (i.e. has a statistical association with) the response variable.

The relationship between  $\delta^{15}\text{N}-\text{NO}_x^-$  and  $\delta^{18}\text{O}-\text{NO}_x^-$  was explored using Pearson's correlation.

We checked whether patterns in consumer  $\delta^{15}\text{N}$  values might be owing to shifts in trophic level across land-use gradients rather than variation in the isotopic composition of nitrogen pools. A 2-end-member mixing model of  $\delta^{13}\text{C}$  autotroph values was used to determine the mix of basal resources potentially contributing to consumer nutrition (Phillips 2012). The  $\delta^{13}\text{C}$  of *P. australis* ( $\delta^{13}\text{C}$  -30.2 to -24.9) and either seagrass ( $\delta^{13}\text{C}$  -25.88 to -10.43) or green filamentous algae ( $\delta^{13}\text{C}$  -32.7 to -12.6) were used in mixing models. Seagrass and green filamentous algae did not always co-occur. Seagrass was preferred in mixing models to maximize the range of  $\delta^{13}\text{C}$  values; green filamentous algae were used for estuaries with no seagrass. The source mixtures identified with mixing models run using  $\delta^{13}\text{C}$  values were applied to  $\delta^{15}\text{N}$  values of sources to provide an estimate of the  $\delta^{15}\text{N}$  of a possible nutritional base for fish ( $\delta^{15}\text{N}_{\text{basal mix}}$ ). While there are likely to be other autotrophic sources contributing to fish nutrition in these estuaries, the autotrophs used represented a range of  $\delta^{13}\text{C}$  values to facilitate modelling a basal mix that could be used to calculate differences between consumer  $\delta^{15}\text{N}$  values and  $\delta^{15}\text{N}$  values of a potential food source (i.e.  $\delta^{15}\text{N}_{(\text{consumer} - \text{basal mix})}$ ) to estimate relative trophic position of the consumers. Linear mixed models were used to regress values of  $\delta^{15}\text{N}_{(\text{consumer} - \text{basal mix})}$  against the fixed land use predictors of the best mixed models identified using  $\text{AIC}_w$ , with site and year included

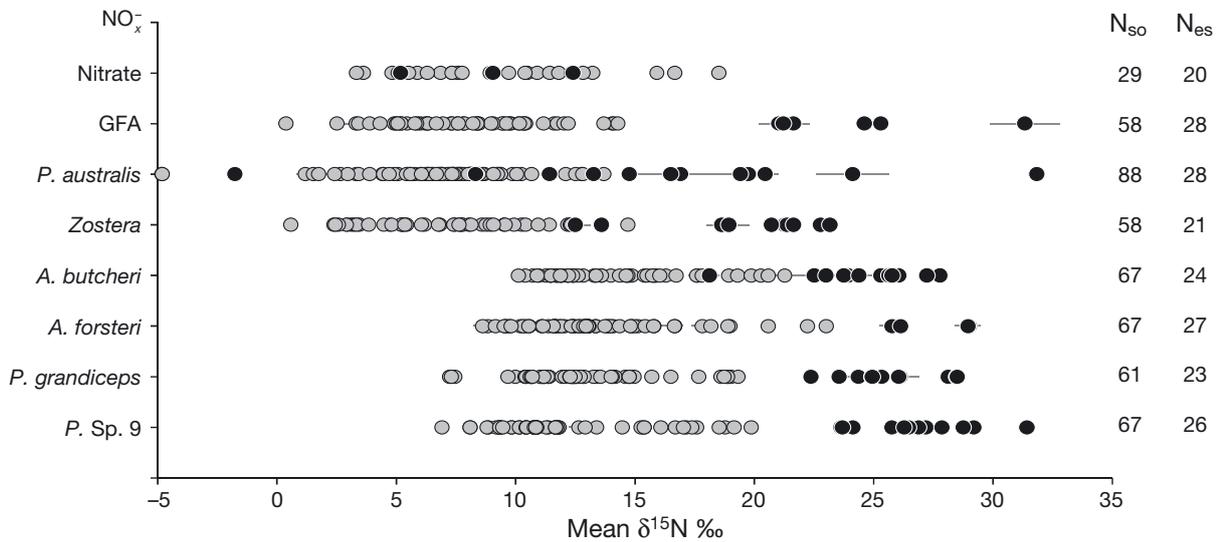


Fig. 2. Mean  $\delta^{15}\text{N} \pm \text{SE}$  (‰) values of green filamentous algae (GFA), *Phragmites australis*, *Zostera* spp., black bream *Acanthopagrus butcheri*, yelloweye mullet *Aldrichetta forsteri*, flathead gudgeon *Philypnodon grandiceps* and eastern bluespot goby *Pseudogobius* sp. 9 taken during this study. Each point represents one particular sampling occasion (i.e. year–season combination) and the number of these ( $N_{so}$ ) is given on the right hand side. The number of estuaries sampled is also presented ( $N_{es}$ ) and  $\text{NO}_x^-$  data are presented; however, only a single measurement was taken on each particular sampling occasion. Estuaries with (●) and without (○) point source inputs from a wastewater treatment plant are shown. A total of 2 estuaries each sampled 6 times receive direct treated wastewater inputs

as random effects. Model performance was assessed using 10-fold cross-validation (Wang et al. 2014).

## RESULTS

The  $\delta^{15}\text{N}$  values of  $\text{NO}_x^-$ , autotrophs and fish ranged considerably among the estuaries studied, with values from  $\sim 3.3$  to  $18.5\text{‰}$  for  $\text{NO}_x^-$ ,  $-4.8$  to  $31.8\text{‰}$  for the autotrophs and  $6.9$  to  $31.4\text{‰}$  for the consumers (Fig. 2). The 2 sites subject to point source treated wastewater discharge had high  $\delta^{15}\text{N}$  values, which were consistent among sampling events (Fig. 2). The  $\delta^{15}\text{N}$  of black bream *Acanthopagrus butcheri* and yelloweye mullet *Aldrichetta forsteri* became higher as catchments became more urbanized.  $\text{AIC}_w$  identified models containing the catchment urbanization predictor as the best among the assessed models for describing patterns in  $\delta^{15}\text{N}$  of these species (Table 1, Fig. 3). These models had good predictive performance:  $R^2_{cv} = 0.82$  for *A. butcheri* and  $R^2_{cv} = 0.70$  for *A. forsteri*.  $W_{var}$  provided further evidence that catchment urbanization was a good predictor of  $\delta^{15}\text{N}$  of *A. butcheri* (catchment urbanization  $W_{var} = 0.81$ ) and *A. forsteri* (catchment urbanization  $W_{var} = 0.94$ ).

Table 1. Relative performance of best linear mixed models fit to ln-transformed  $\delta^{15}\text{N}$  response data. Model performance presented as relative probability weights based on Akaike's information criterion corrected for small sample size converted to AIC weights ( $\text{AIC}_w$ ) for the best model. Cross-validated  $R^2$  ( $R^2_{cv}$ ) values are also presented

Response	Best model	$\text{AIC}_w$	$R^2_{cv}$
$\text{NO}_x^-$	Site + year + tide	0.451	0.057
<b>Plants</b>			
Green filamentous algae	Site + year	0.492	0.033
<i>Phragmites australis</i>	Site + year	0.883	0.050
<i>Zostera</i> spp.	Site + year + season	0.938	0.448
<b>Fish</b>			
<i>Acanthopagrus butcheri</i>	Site + year + urbanization	0.365	0.819 <sup>a</sup>
<i>Aldrichetta forsteri</i>	Site + year + urbanization	0.495	0.700 <sup>a</sup>
<i>Philypnodon grandiceps</i>	Site + year	0.926	0.660
<i>Pseudogobius</i> sp. 9	Site + year	0.527	0.607

<sup>a</sup>Values of  $R^2_{cv}$  indicating good predictive performance

The  $\delta^{15}\text{N}$  values of *Phragmites australis*, green filamentous algae, flathead gudgeon *Philypnodon grandiceps* and eastern bluespot goby *Pseudogobius* sp. 9 were not influenced by catchment land use, tidal exchange or season.  $\text{AIC}_w$  identified the null model containing only the random effects site and year as the best among the models assessed (Table 1).  $W_{var}$  also indicated that catchment land use did not influence the  $\delta^{15}\text{N}$  of these taxa (Table 2, Fig. 3, Table S52 in Supplement 4).

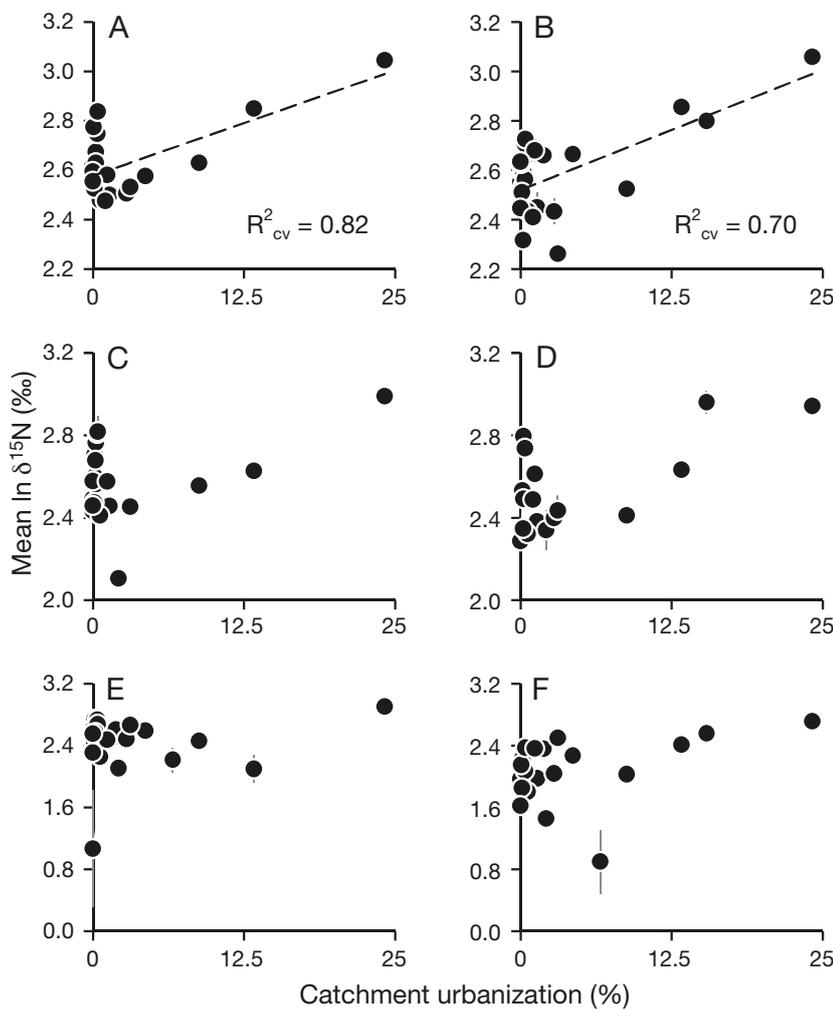


Fig. 3. Mean  $\ln \delta^{15}\text{N} \pm \text{SE}$  (‰) versus catchment urbanization (%) for (A) black bream *Acanthopagrus butcheri*, (B) yelloweye mullet *Aldrichetta forsteri*, (C) flathead gudgeon *Philypnodon grandiceps*, (D) eastern bluespot goby *Pseudogobius* sp. 9, (E) *Phragmites australis* and (F) green filamentous algae.  $\ln$ -transformed  $\delta^{15}\text{N}$  values were averaged over years and seasons if sampled multiple times from a given estuary. Modelled responses (dashed lines) and cross-validated  $R^2$  ( $R^2_{cv}$ ) presented for models with good predictive performance

Relationships between the  $\delta^{15}\text{N}$  of *A. butcheri* and *A. forsteri* and catchment urbanization were not due to shifts in trophic position. The model that included catchment urbanization was the best for describing  $\delta^{15}\text{N}$  of *A. butcheri* and *A. forsteri* (Table 1), but had very low predictive capacity for  $\delta^{15}\text{N}_{(\text{consumer} - \text{basal mix})}$  for these species (cross-validated  $R^2 < 0.1$ ).

The  $\delta^{15}\text{N}$  values of *Zostera* spp. were higher in spring than autumn but were not influenced by catchment land use. The model containing season best described patterns of  $\delta^{15}\text{N}$  in seagrass ( $\text{AIC}_w = 0.94$ ; Table 1) and this model had moderate predictive performance ( $R^2_{cv} = 0.45$ ).  $W_{\text{var}}$  pro-

Table 2. Relative importance of each predictor variable used in linear mixed models, in explaining patterns of  $\ln$ -transformed  $\delta^{15}\text{N}$  responses. Adjusted variable weights ( $W_{\text{var}}$ ; probability that a given variable is included in the best model variant) for each variable are shown. See Table S5 in Supplement 4 at [www.int-res.com/articles/suppl/542p025\\_supp.pdf](http://www.int-res.com/articles/suppl/542p025_supp.pdf) for  $\text{AIC}_w$  values for each model variant, and prior and posterior odds and odds ratios for explanatory variables. Tide: tidal exchange; Ag: catchment used for intensive agriculture; Urb: urbanized catchment; \*interaction terms

Response	Model terms								
	Season	Tide	Ag (%)	Urb (%)	Season* Tide	Season* Ag	Season* Urb	Tide* Ag	Tide* Urb
$\text{NO}_x^-$	0.165	0.709	0.010	0.084	0.373	0.001	0.001	0.001	0.017
<b>Plants</b>									
Green filamentous algae	0.503	0.101	0.065	0.153	0.001	0.001	0.010	<0.001	<0.001
<i>Phragmites australis</i>	0.117	0.015	0.033	0.028	<0.001	<0.001	0.001	<0.001	<0.001
<i>Zostera</i> spp.	0.996 <sup>a</sup>	0.006	0.008	0.104	<0.001	0.001	0.025	<0.001	<0.001
<b>Fish</b>									
<i>Acanthopagrus butcheri</i>	0.547	0.002	0.003	0.807 <sup>a</sup>	<0.001	<0.001	0.011	<0.001	<0.001
<i>Aldrichetta forsteri</i>	0.509	0.002	0.003	0.942 <sup>a</sup>	<0.001	<0.001	0.079	<0.001	<0.001
<i>Philypnodon grandiceps</i>	0.039	0.002	0.021	0.078	<0.001	0.001	<0.001	<0.001	<0.001
<i>Pseudogobius</i> sp. 9	0.077	0.015	0.012	0.628	<0.001	<0.001	0.003	<0.001	<0.001

<sup>a</sup>Values of  $W_{\text{var}}$  indicating predictors that have a statistical association with the response variable

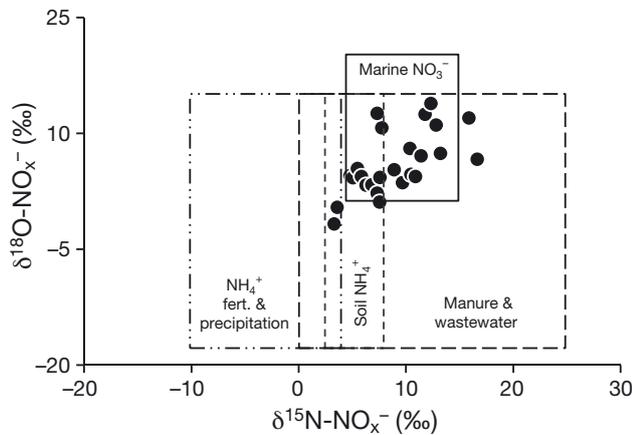


Fig. 4. Dual  $\delta^{15}\text{N-NO}_x^-$  and  $\delta^{18}\text{O-NO}_x^-$  plots for estuaries not directly subject to point source treated wastewater discharge. Each point represents a particular site and sampling occasion (i.e. year–season combination). Boxes outline ranges of  $\delta^{15}\text{N}$  and  $\delta^{18}\text{O}$  isotopic signatures of different inorganic nitrogen sources (e.g. Kendall et al. 2007). The ‘soil  $\text{NH}_4^+$ ’ box is completely encompassed by the ‘manure & wastewater’ box, and both of these boxes overlap with the ‘ $\text{NH}_4^+$  fertilizer & precipitation’ box.  $N = 25$

vided further evidence that season was a strong predictor of  $\delta^{15}\text{N}$  of *Zostera* spp. (season  $W_{\text{var}} > 0.99$ ; Table 2, Table S5 in Supplement 4).

The model containing tide best described patterns of  $\delta^{15}\text{N}$  of  $\text{NO}_x^-$  (Table 1), although this model had very low predictive capacity ( $R^2_{\text{cv}} = 0.06$ ; Table 1) and  $W_{\text{var}}$  provided little indication that predictors were important for describing  $\delta^{15}\text{N}$  of  $\text{NO}_x^-$  (Table 2, Table S5 in Supplement 4). Values of  $\delta^{15}\text{N-NO}_x^-$  and  $\delta^{18}\text{O-NO}_x^-$  were positively correlated (Pearson’s correlation  $R = 0.62$ ,  $p = 0.001$ ) and within ranges in which nitrogen sources were likely wastewater and manure, marine  $\text{NO}_3^-$  or soil  $\text{NH}_4^+$  (Fig. 4).

## DISCUSSION

There has been limited use of nitrogen stable isotopes ( $\delta^{15}\text{N}$ ) for detecting anthropogenic nitrogen inputs to estuarine ecosystems among catchments distributed over hundreds of kilometres. This study showed positive responses of the  $\delta^{15}\text{N}$  values of 2 consumers—black bream *Acanthopagrus butcheri* and yelloweye mullet *Aldrichetta forsteri*—to the proportion of the catchment subject to urbanization. The  $\delta^{15}\text{N}$  of  $\text{NO}_x^-$ , autotrophs and smaller-bodied consumers—flathead gudgeon *Philypnodon grandiceps* and eastern bluespot goby *Pseudogobius* sp. 9—did not respond clearly to catchment land use over the scale investigated.

### High $\delta^{15}\text{N}$ values near point sources of treated wastewater

The  $\delta^{15}\text{N}$  values of autotrophs and consumers were high at estuaries subject to point source wastewater treatment plant discharge. High  $\delta^{15}\text{N}$  values of biota and inorganic nitrogen are commonly recorded near discharge points from wastewater treatment plants (e.g. deBruyn & Rasmussen 2002, Connolly et al. 2013, Hood et al. 2014). Nitrogen discharged from wastewater treatment plants has high  $\delta^{15}\text{N}$  values due to the enriching processes of  $\text{NH}_4^+$  volatilization and  $\text{NO}_3^-$  denitrification (Desimone & Howes 1996). Plants and fish from the Little and Werribee rivers consistently had high  $\delta^{15}\text{N}$  values (up to  $\sim 30\%$ ; Fig. 2), which were not unexpected given the high  $\delta^{15}\text{N-NO}_3^-$  values previously observed adjacent to the Western Treatment Plant (Wong et al. 2014, 2015). High  $\delta^{15}\text{N-NO}_3^-$  values have been observed in surface waters throughout the Werribee River estuary ( $\sim 15$  to  $28\%$ ; Wong et al. 2014) and ground waters on the western side of this estuary adjacent to the treatment plant ( $\sim 33\%$ ; Wong et al. 2015). Connolly et al. (2013) also observed high  $\delta^{15}\text{N}$  values of  $\sim 20$  to  $25\%$  in green algae, crabs and planktivorous fish sampled from coastal waters  $< 5$  km from point sources of treated wastewater in Adelaide, South Australia. Hood et al. (2014) similarly observed  $\delta^{15}\text{N}$  values up to  $24\%$  in macrophytes  $< 5$  km downstream of wastewater treatment plants on the Grand River, Ontario, Canada.

### Catchment land use and $\delta^{15}\text{N}$ of biota

We observed positive relationships between catchment urbanization and the  $\delta^{15}\text{N}$  of black bream *A. butcheri* and yelloweye mullet *A. forsteri* over the catchments investigated here (Tables 1 & 2, Fig. 3). Other studies have documented trends of higher  $\delta^{15}\text{N}$  of biota with increasing urbanization of catchments (see e.g. Bannon & Roman 2008, Olsen et al. 2010) and population-based measures of diffuse human wastewater inputs (see e.g. Cole et al. 2004, 2005, Bannon & Roman 2008, Hoffman et al. 2012) in estuaries. However, these studies were typically conducted at local or catchment-specific spatial scales (but see Cole et al. 2004, Hoffman et al. 2012).

Catchment urbanization (as a percentage of total catchment area) in our study ranged from 0 to 25%, which is a smaller range than that investigated in estuarine systems by Bannon & Roman (2008; 0 to 65%) or the range of urbanization of shorelines ad-

adjacent to the coastal study locations of Olsen et al. (2010; 0 to 80%). However, despite the smaller urbanization range in our study, increases in the  $\delta^{15}\text{N}$  of 2 consumers with increasing catchment urbanization were observed, particularly at levels of urbanization exceeding ~10% of the catchment (Fig. 3). Bannon & Roman (2008) and Olsen et al. (2010) also observed positive relationships between the  $\delta^{15}\text{N}$  of invertebrate and fish consumers and catchment urbanization. The 2 estuaries with the highest degree of catchment urbanization and highest  $\delta^{15}\text{N}$  values of those estuaries analysed in modelling of relationships between catchment land use and  $\delta^{15}\text{N}$  do not have wastewater treatment plants that discharge to surface waters in their catchments (Geoscience Australia, National Wastewater Treatment Plant Database at [www.ga.gov.au/metadata-gateway/metadata/record/gcat\\_74625](http://www.ga.gov.au/metadata-gateway/metadata/record/gcat_74625) [accessed August 2015]). The 3 most urbanized estuaries had similar proportions of intensive agriculture in their catchments (Table S1 in Supplement 1).

There was considerable unexplained variability in relationships between catchment urbanization and  $\delta^{15}\text{N}$  of *A. butcheri* and *A. forsteri*, as well as other biota, at levels of catchment urbanization <10% (Fig. 3). It is expected that at low levels of catchment urbanization the dominance of this land use on the isotopic source pool is diminished as mixing with nitrogen derived from alternative land uses increases. Estuaries within more highly urbanized catchments may also be more tightly coupled to land use in their catchments due to increased impervious surfaces, which potentially aids the transport of water and nutrients through catchments to estuaries (Bowen & Valiela 2008). Outside of urban areas, many households use septic tanks rather than a sewage network in Victoria, which may also explain some of the higher than expected  $\delta^{15}\text{N}$  values at very low levels of catchment urbanization (Steffy & Kilham 2004). Strong relationships between dwelling densities and  $\delta^{15}\text{N}$  in estuaries have been observed when catchments have been exclusively serviced by septic tanks (see e.g. McClelland & Valiela 1998); however, information on septic infrastructure is not readily available in Victoria. Lower  $\delta^{15}\text{N}$  values have been observed as sewer coverage in urban catchments increases and wastewater is transported to treatment plants in other catchments (Steffy & Kilham 2004, Barnes & Raymond 2010). In this study, the 2 estuaries with the highest  $\delta^{15}\text{N}$  values—Watsons Creek and Kororoit Creek—were in the most urbanized catchments (Table S1 in Supplement 1) that are fully connected to sewage networks and do not con-

tain wastewater treatment plants that discharge to surface waters (Geoscience Australia, National Wastewater Treatment Plant Database at [www.ga.gov.au/metadata-gateway/metadata/record/gcat\\_74625](http://www.ga.gov.au/metadata-gateway/metadata/record/gcat_74625) [accessed August 2015]). It is also possible that high levels of intensive agriculture in some catchments with low levels of urbanization increased  $\delta^{15}\text{N}$  values in some estuaries; however, this did not occur systematically because models containing urbanization by intensive agriculture interaction terms did not perform well (Tables 1 & 2).

The proportion of the catchment used for intensive agriculture did not describe patterns of  $\delta^{15}\text{N}$  of  $\text{NO}_x^-$ , autotrophs or consumers and was not selected in any models of best fit (Tables 1 & 2). Positive relationships between intensive agricultural land use and the  $\delta^{15}\text{N}$  of aquatic biota have been previously observed in freshwater streams in agricultural landscapes (Anderson & Cabana 2005, Diebel & Vander Zanden 2009). Studies have attributed high  $\delta^{15}\text{N}$  of stream biota to the contribution of manure to agricultural nitrogen pools and to biogeochemical transformations (e.g. denitrification) of isotopically light inorganic fertilizers during transport to, or within, streams (Anderson & Cabana 2005, Diebel & Vander Zanden 2009). A lack of relationships between intensive agriculture and  $\delta^{15}\text{N}$  in our estuaries may result from the mixing of nitrogen sources within the catchments studied, or non-uniformity in biogeochemical transformations of nitrogen during transport from agricultural sources to estuaries, as well as the confounding effect of marine sources of nitrogen. Valiela et al. (2014) found that within-estuary nitrogen transformations obscured effects of catchment vegetation cover that were observed on the  $\delta^{15}\text{N}$  of SPOM in freshwater reaches of stream networks in 8 catchments. Inorganic nitrogen  $\delta^{15}\text{N}$  and  $\delta^{18}\text{O}$  values from estuaries in this study were within ranges where multiple sources were possible (Kendall et al. 2007, Xue et al. 2009). However, there was a strong positive correlation between  $\delta^{15}\text{N}\text{-NO}_x^-$  and  $\delta^{18}\text{O}\text{-NO}_x^-$  ( $R = 0.62$ ; Fig. 4), suggesting nitrogen transformations may have been occurring at some sites (Kendall et al. 2007, Kaushal et al. 2011). Positive correlations between  $\delta^{15}\text{N}\text{-NO}_3^-$  and  $\delta^{18}\text{O}\text{-NO}_3^-$  have been observed elsewhere and used to infer the removal of catchment-derived nitrogen via denitrification (Kaushal et al. 2011). The large size of some catchments in our study and their distribution across hundreds of kilometres increase the potential for both mixing of isotopic sources and variation in location-specific characteristics, such as geology and hydrology, that may affect nitrogen transformations.

Nitrogen loads to estuaries are high in Victorian catchments where land use is dominated by intensive agriculture (Woodland et al. 2015), indicating agricultural land uses are contributing to the nitrogen pool of at least some Victorian estuaries. The lack of relationships between  $\delta^{15}\text{N}$  and intensive agriculture in our study does not mean that nitrogen from agricultural land use is not entering estuaries. It suggests that either  $\delta^{15}\text{N}$  is not robust enough to facilitate detection of agricultural nitrogen in estuaries situated in catchments distributed over hundreds of kilometres, or broad categories of agricultural land use (in our case, aggregated modified pasture, intensive animal production and intensive horticulture) are not sufficient for describing potential effects of agriculture on isotopic source pools. However, analyses of the 3 agricultural land use types as individual predictors did not change modelling results. More detailed land use data including fertilizer application rates, crop rotations and livestock densities may be required to identify links between agricultural intensity and  $\delta^{15}\text{N}$  in estuaries. Information on catchment soils and groundwater inputs to estuaries may also be required to interpret  $\delta^{15}\text{N}$  values of estuarine biota and detect potentially subtle effects of agricultural land uses on the isotopic composition of nitrogen pools. However, such detailed land use, soil and groundwater data, and flow and water nutrient concentration measurements for estimating nutrient loads, are not available in many locations, including Victoria, and issues of consistency of data arise during coast-wide investigation.

#### Variable responses of $\delta^{15}\text{N}$ to catchment urbanization

Relationships between catchment urbanization and  $\delta^{15}\text{N}$  were not observed for all taxa examined. Only  $\delta^{15}\text{N}$  values of 2 of the large-bodied adult fish—black bream *A. butcheri* and yelloweye mullet *A. forsteri*—demonstrated positive relationships to catchment urbanization. The  $\delta^{15}\text{N}$  values of  $\text{NO}_x^-$ , autotrophs and small-bodied fish did not respond statistically to catchment land use, although patterns were qualitatively similar to those of larger-bodied fish (Fig. 3) and the  $\delta^{15}\text{N}$  values of all fish species were correlated ( $R^2 > 0.6$ ; Supplement 5 at [www.int-res.com/articles/suppl/m542p025\\_supp.pdf](http://www.int-res.com/articles/suppl/m542p025_supp.pdf)).

The stable isotope composition of consumer tissues reflects the assimilation of prey over time and the kinetics of internal metabolic processes. Larger-bodied consumers often require longer isotopic equi-

libration periods than autotrophs or small-bodied consumers because of slower somatic growth rates and scaling effects of body mass on metabolism (Clarke & Johnston 1999, Woodward et al. 2008). The stronger relationships between urbanization and  $\delta^{15}\text{N}$  of large-bodied fish consumers than small-bodied fish or autotrophs may reflect the time-averaging effect of longer equilibration times. Temporal variability in hydrological inputs, tidal exchange and nitrogen cycling can alter  $\delta^{15}\text{N}$  of estuarine nitrogen pools over short periods (e.g. daily, weekly or monthly). The  $\delta^{15}\text{N}$  of *A. butcheri* and *A. forsteri* may also provide a spatially integrated signal of catchment-derived nitrogen to the estuary, as these species are relatively mobile, omnivorous feeders, likely to feed in multiple habitats within estuaries and integrate the isotopic signatures of a variety of food sources. The  $\delta^{15}\text{N}$  of small-bodied, cryptic fish such as flathead gudgeon *P. grandiceps* or eastern blue-spot goby *Pseudogobius* sp. 9 and autotrophs may be more influenced by patch-scale processes that obscure ecosystem-scale patterns. The  $\delta^{15}\text{N}$  of *A. butcheri* and *A. forsteri* may have reduced signal-to-noise ratios and provide a better indicator of land use effects on estuarine nitrogen isotopic pools (Fertig et al. 2009, Hoffman et al. 2012) than either the  $\text{NO}_x^-$  or the other organisms with faster tissue turnover and lower mobility. However, this is inconsistent with other work that has demonstrated small-bodied larval fish are effective indicators of catchment-derived anthropogenic nutrient inputs (Hoffman et al. 2012). The local affinity of small-bodied fish in our study for certain habitats and depths means local isotopic conditions may prevail over integrated system-wide conditions.

Fish diet may also contribute to the stronger relationships between urbanization and the  $\delta^{15}\text{N}$  of larger- than smaller-bodied fish. Isotopically heavy nitrogen from urban land use delivered to estuaries by runoff may be incorporated into food webs through pelagic pathways. *A. butcheri* and *A. forsteri* are demersal species with higher mobility than the closely benthic associated *P. grandiceps* and *Pseudogobius* sp. 9. This can potentially increase encounters of planktonic prey with isotopic values reflective of phytoplankton, which was not measured in this study. Black bream also feed on filter-feeding polychaetes and bivalves, which would likely have isotopic values reflective of phytoplankton.

The higher plants studied here—*Phragmites australis* and *Zostera* spp.—have the capacity to fractionate the dissolved inorganic nitrogen pool on uptake (Campbell & Fourqurean 2009) and during

translocation of nitrogen from old to new tissues, such as from rhizomes to shoots (Choi et al. 2005). The  $\delta^{15}\text{N}$  of seagrasses can also be influenced by bacterially mediated nitrogen fixation in the rhizosphere (Welsh 2000) or uptake of isotopically light, newly mineralized nitrogen within sediments (Cook et al. 2015). Such biogeochemical processes may, in addition to relatively fast tissue turnover, contribute to the decoupling of *P. australis* and *Zostera* spp.  $\delta^{15}\text{N}$  values from catchment land use in this study (Tables 1 & 2). Christiaen et al. (2014) also found little evidence for a relationship between seagrass  $\delta^{15}\text{N}$  and catchment urbanization, which they measured as population density. Taxonomy and latitude were the strongest influences on seagrass  $\delta^{15}\text{N}$  at the global scale (Christiaen et al. 2014). We sampled 2 *Zostera* spp. of seagrass—*Z. muelleri* and *Z. nigricaulis*—from similar latitudes, within the range 38.86 to 37.57°S (Fig. 1). Despite the restriction of taxonomy and latitude, we also failed to detect relationships between seagrass  $\delta^{15}\text{N}$  and urbanization of catchments distributed across a coastline.

### CONCLUSIONS

The  $\delta^{15}\text{N}$  values of large-bodied consumers increased with catchment urbanization, but these relationships were absent when <10% of the catchment was urbanized. The lack of relationships between  $\delta^{15}\text{N}$  values in estuaries and intensive agriculture in catchments suggests that  $\delta^{15}\text{N}$  cannot overcome mixing of isotopic sources or non-uniformity in nitrogen transformations among catchments distributed over hundreds of kilometres, or that detailed agricultural land use data are required to establish patterns. The  $\delta^{15}\text{N}$  values of large-bodied consumers may provide useful indicators of the incorporation of non-point source anthropogenic nitrogen into estuarine food webs in urbanized catchments, because of long tissue turnover times that reduce signal-to-noise ratios. These taxa can be used to identify dominant sources of nitrogen entering estuarine food webs in highly urbanized catchments, which may provide a basis from which to evaluate changes in urban nutrient inputs and guide management actions among catchments. The  $\delta^{15}\text{N}$  values of plants or animals with more rapid tissue turnover may be more useful for tracking changes at particular sites through time, or comparing sites dispersed across smaller spatial scales where variation in confounding factors influencing nitrogen isotopic pools (e.g. hydrology and geology) are less pronounced.

**Acknowledgements.** Thanks to T. Daniel, D. Hartwell, J. Williams and R. Ayres for assistance in the field and laboratory. Thanks to J. Yen for assistance with statistics. Funding was provided by Melbourne Water, the Victorian Investment Fund and an Australian Research Council grant (LP110100040) to P.R., P.L.M.C. and R.M. F.Y.W. is supported by an Australian Postgraduate Award. Work presented here complies with Australian law.

### LITERATURE CITED

- Anderson C, Cabana G (2005)  $\delta^{15}\text{N}$  in riverine food webs: effects of N inputs from agricultural watersheds. *Can J Fish Aquat Sci* 62:333–340
- Bannon RO, Roman CT (2008) Using stable isotopes to monitor anthropogenic nitrogen inputs to estuaries. *Ecol Appl* 18:22–30
- Barnes RT, Raymond PA (2010) Land-use controls on sources and processing of nitrate in small watersheds: insights from dual isotopic analysis. *Ecol Appl* 20: 1961–1978
- Bowen JL, Valiela I (2008) Using  $\delta^{15}\text{N}$  to assess coupling between watersheds and estuaries in temperate and tropical regions. *J Coast Res* 24:804–813
- Burns DA, Boyer EW, Elliott EM, Kendall C (2009) Sources and transformations of nitrate from streams draining varying land uses: evidence from dual isotope analysis. *J Environ Qual* 38:1149–1159
- Campbell JE, Fourqurean JW (2009) Interspecific variation in the elemental and stable isotope content of seagrasses in South Florida. *Mar Ecol Prog Ser* 387:109–123
- Casciotti KL, Sigman DM, Hastings MG, Bohlke JK, Hilkert A (2002) Measurement of the oxygen isotopic composition of nitrate in seawater and freshwater using the denitrifier method. *Anal Chem* 74:4905–4912
- Choi WJ, Chang SX, Ro HM (2005) Seasonal changes of shoot nitrogen concentrations and  $^{15}\text{N}/^{14}\text{N}$  ratios in common reed in a constructed wetland. *Commun Soil Sci Plan* 36:2719–2731
- Christiaen B, Bernard RJ, Mortazavi B, Cebrian J, Ortmann AC (2014) The degree of urbanization across the globe is not reflected in the  $\delta^{15}\text{N}$  of seagrass leaves. *Mar Pollut Bull* 83:440–445
- Clarke A, Johnston NM (1999) Scaling of metabolic rate with body mass and temperature in teleost fish. *J Anim Ecol* 68:893–905
- Cole ML, Valiela I, Kroeger KD, Tomasky GL and others (2004) Assessment of a  $\delta^{15}\text{N}$  isotopic method to indicate anthropogenic eutrophication in aquatic ecosystems. *J Environ Qual* 33:124–132
- Cole ML, Kroeger KD, McClelland JW, Valiela I (2005) Macrophytes as indicators of land-derived wastewater: application of a  $\delta^{15}\text{N}$  method in aquatic systems. *Water Resour Res* 41:W01014, doi:10.1029/2004WR003269
- Cole ML, Kroeger KD, McClelland JW, Valiela I (2006) Effects of watershed land use on nitrogen concentrations and  $\delta^{15}\text{N}$  nitrogen in groundwater. *Biogeochemistry* 77: 199–215
- Connolly RM, Gorman D, Hindell JS, Kildea TN, Schlacher TA (2013) High congruence of isotope sewage signals in multiple marine taxa. *Mar Pollut Bull* 71:152–158
- Cook PLM, Evrard V, Woodland RJ (2015) Factors controlling nitrogen fixation in temperate seagrass beds. *Mar Ecol Prog Ser* 525:41–51

- Costanzo SD, Udy J, Longstaff B, Jones A (2005) Using nitrogen stable isotope ratios ( $\delta^{15}\text{N}$ ) of macroalgae to determine the effectiveness of sewage upgrades: changes in the extent of sewage plumes over four years in Moreton Bay, Australia. *Mar Pollut Bull* 51:212–217
- deBruyn AMH, Rasmussen JB (2002) Quantifying assimilation of sewage-derived organic matter by riverine benthos. *Ecol Appl* 12:511–520
- Desimone LA, Howes BL (1996) Denitrification and nitrogen transport in a coastal aquifer receiving wastewater discharge. *Environ Sci Technol* 30:1152–1162
- Diebel MW, Vander Zanden MJ (2009) Nitrogen stable isotopes in streams: effects of agricultural sources and transformations. *Ecol Appl* 19:1127–1134
- Erisman JW, Sutton MA, Galloway J, Klimont Z, Winiwarter W (2008) How a century of ammonia synthesis changed the world. *Nat Geosci* 1:636–639
- Fertig B, Carruthers TJB, Dennison WC, Jones AB, Pantus F, Longstaff B (2009) Oyster and macroalgae bioindicators detect elevated  $\delta^{15}\text{N}$  in Maryland's coastal bays. *Estuar Coast* 32:773–786
- Fry B (2006) *Stable isotope ecology*. Springer, New York, NY
- Galloway JN, Aber JD, Erisman JW, Seitzinger SP, Howarth RW, Cowling EB, Cosby BJ (2003) The nitrogen cascade. *Bioscience* 53:341–356
- Hesslein RH, Hallard KA, Ramlal P (1993) Replacement of sulfur, carbon, and nitrogen in tissue of growing broad whitefish (*Coregonus nasus*) in response to a change in diet traced by  $\delta^{34}\text{S}$ ,  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$ . *Can J Fish Aquat Sci* 50:2071–2076
- Hoffman JC, Kelly JR, Peterson GS, Cotter AM, Starry MA, Sierszen ME (2012) Using  $\delta^{15}\text{N}$  in fish larvae as an indicator of watershed sources of anthropogenic nitrogen: response at multiple spatial scales. *Estuar Coast* 35:1453–1467
- Hood JLA, Taylor WD, Schiff SL (2014) Examining the fate of WWTP effluent nitrogen using  $\delta^{15}\text{N-NH}_4^+$ ,  $\delta^{15}\text{N-NO}_3^-$  and  $\delta^{15}\text{N}$  of submersed macrophytes. *Aquat Sci* 76:243–258
- Kaushal SS, Groffman PM, Band LE, Elliott EM, Shields CA, Kendall C (2011) Tracking nonpoint source nitrogen pollution in human-impacted watersheds. *Environ Sci Technol* 45:8225–8232
- Kendall C, Elliott EM, Wankel SD (2007) Tracing anthropogenic inputs of nitrogen to ecosystems. Lajtha K, Michener RH (eds) *Stable isotopes in ecology and environmental science*. Blackwell, Malden, MA, p 375–449
- McClelland JW, Valiela I (1998) Linking nitrogen in estuarine producers to land-derived sources. *Limnol Oceanogr* 43:577–585
- Olsen YS, Fox SE, Kinney EL, Teichberg M, Valiela I (2010) Differences in urbanization and degree of marine influence are reflected in  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  of producers and consumers in seagrass habitats of Puerto Rico. *Mar Environ Res* 69:198–206
- Phillips DL (2012) Converting isotope values to diet composition: the use of mixing models. *J Mammal* 93:342–352
- Pinnegar JK, Polunin NVC (1999) Differential fractionation of delta C-13 and delta N-15 among fish tissues: implications for the study of trophic interactions. *Funct Ecol* 13:225–231
- Pitt KA, Connolly RM, Maxwell P (2009) Redistribution of sewage-nitrogen in estuarine food webs following sewage treatment upgrades. *Mar Pollut Bull* 58:573–580
- R Core Team (2014) R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna. Available at [www.r-project.org/](http://www.r-project.org/)
- Sigman DM, Casciotti KL, Andreani M, Barford C, Galanter M, Bohlke JK (2001) A bacterial method for the nitrogen isotopic analysis of nitrate in seawater and freshwater. *Anal Chem* 73:4145–4153
- Steffy LY, Kilham SS (2004) Elevated  $\delta^{15}\text{N}$  in stream biota in areas with septic tank systems in an urban watershed. *Ecol Appl* 14:637–641
- Stein JL, Hutchinson MF, Stein JA (2014) A new stream and nested catchment framework for Australia. *Hydrol Earth Syst Sci* 18:1917–1933
- Symonds MRE, Moussalli A (2011) A brief guide to model selection, multimodel inference and model averaging in behavioural ecology using Akaike's information criterion. *Behav Ecol Sociobiol* 65:13–21
- Triba MN, Le Moyec L, Amathieu R, Goossens C and others (2015) PLS/OPLS models in metabolomics: the impact of permutation of dataset rows on the K-fold cross-validation quality parameters. *Mol Biosyst* 11:13–19
- Valiela I, Bartholomew M, Giblin A, Tucker J and others (2014) Watershed deforestation and down-estuary transformations alter sources, transport, and export of suspended particles in Panamanian mangrove estuaries. *Ecosystems* 17:96–111
- Vander Zanden MJ, Vadeboncoeur Y, Diebel MW, Jeppesen E (2005) Primary consumer stable nitrogen isotopes as indicators of nutrient source. *Environ Sci Technol* 39:7509–7515
- Wagenmakers EJ, Farrell S (2004) AIC model selection using Akaike weights. *Psychon Bull Rev* 11:192–196
- Wang P, Tamilselvan P, Hu C (2014) Health diagnostics using multi-attribute classification fusion. *Eng Appl Artif Intel* 32:192–202
- Wankel SD, Kendall C, Paytan A (2009) Using nitrate dual isotopic composition ( $\delta^{15}\text{N}$  and  $\delta^{18}\text{O}$ ) as a tool for exploring sources and cycling of nitrate in an estuarine system: Elkhorn Slough, California. *J Geophys Res* 114:G01011, doi:10.1029/2008JG000729
- Warry FY, Reich P, Hindell JS, McKenzie J, Pickworth A (2013) Using new electrofishing technology to amp-up fish sampling in estuarine habitats. *J Fish Biol* 82:1119–1137
- Welsh DT (2000) Nitrogen fixation in seagrass meadows: regulation, plant-bacteria interactions and significance to primary productivity. *Ecol Lett* 3:58–71
- Wong WW, Grace MR, Cartwright I, Cook PLM (2014) Sources and fate of nitrate in a groundwater-fed estuary elucidated using stable isotope ratios of nitrogen and oxygen. *Limnol Oceanogr* 59:1493–1509
- Wong WW, Grace MR, Cartwright I, Cook PLM (2015) Unravelling the origin and fate of nitrate in an agricultural-urban coastal aquifer. *Biogeochemistry* 122:343–360
- Woodland, RJ, Warry FY, Evrard V, Clarke RH, Reich P, Cook PLM (2015) Nitrogen loads explain primary productivity in estuaries at the ecosystem scale. *Limnol Oceanogr* 60:1751–1762
- Woodward G, Papanoniu G, Edwards F, Lauridsen RB (2008) Trophic trickles and cascades in a complex food web: impacts of a keystone predator on stream community structure and ecosystem processes. *Oikos* 117:683–692
- Xue D, Botte J, De Baets B, Accoe F and others (2009) Present limitations and future prospects of stable isotope methods for nitrate source identification in surface- and groundwater. *Water Res* 43:1159–1170