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EXECUTIVE SUMMARY

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Agricultural and urban development can increase run-off and lead to excessive nutrient loadings in fragile coastal environments that are nursery grounds for a diverse array of coastal and estuarine species, as well as other resident organisms. This project investigates the development of bioindicators to strengthen the ability of managers to detect and quantify changes in anthropogenic nitrogen inputs to coastal and estuarine ecosystems by comparing six study sites with different levels of development ranging from pristine through to fully urban. The results show a strong positive relationship between the percent agricultural land in surrounding catchments and total nitrogen (TN) loading to nearshore environments.

Stable nitrogen isotope ratios ($\delta^{15}N$) of primary producers increased with increasing water column dissolved inorganic nitrogen (DIN) concentration and TN loading. The $\delta^{15}N$ value of macroalgae (*Ulva* spp.) was clearly related to wastewater nitrogen loads in coastal environments, thus demonstrating that it is a good indicator of land-derived nutrients around urban watersheds. This relationship was most significant during the algal growth period (spring-summer), suggesting that sampling macroalgae to detect regional differences in isotopic values should be conducted in summer. By contrast, isotope ratios of primary consumers (filter-feeding bivalves) showed no clear relationship to different terrestrial nutrient sources among estuaries. However, there was a clear spatial gradient within individual estuaries, which was decoupled from the isotopic gradients of primary producers.

These results hint at differences in dissolved and particulate nitrogen source pools, and highlight the importance of using complementary components of food webs and high spatial replication to show linkages between watershed land use and chemical markers in biota. The effects of nutrient enrichment were transmitted up the food web, with growth of secondary consumers, *Notolabrus celidotus* (spotties) and *Grahamina nigripenne* (estuarine triplefins) generally enhanced in nutrient-enriched coastal areas. Benthic prey dominated the diets of these fish species, with amphipods and brachyurans being the most important prey items for triplefins and spotties, respectively. However, there were site-specific differences in prey importance and diet diversity. Both triplefins and spotties consumed considerably more diverse prey items at pristine than nutrient-enriched coastal areas. Food web models based on stomach content analyses and dual isotope ratios suggest that there are shifts in the relative importance of the different organic matter sources supporting food structure among the different coastal ecosystems due to nutrient enhancement from land-based activities.

1. INTRODUCTION

1.1 Rationale for study

Nearshore estuarine and marine ecosystems have extremely high primary and secondary productivity and support a diversity of fish and invertebrate species (Beck et al. 2001). These ecosystems are increasingly threatened by anthropogenic activities in the coastal zone and in surrounding catchments. Human activities in watersheds, such as land clearing, application of fertilisers, discharge of human waste, animal production, and fossil fuel combustion, can increase the flux and rate of delivery of terrestrial nutrients to coastal environments (Galloway et al. 2003). This increased availability of nutrients (nitrogen and phosphorus) can, in turn, degrade water quality and profoundly impact recipient aquatic ecosystems (McClelland & Valiela 1998, Bowen & Valiela 2001, Kelly 2001, Deegan et al. 2002, Rabalais 2002). Typical changes in the structure and function of nearshore coastal ecosystems associated with nutrient enrichment include elevated algal production and biomass, altered primary producer communities, and shifts in fish populations (Cloern 2001). Nutrient enrichment from human activities in watersheds is currently recognised as one of the foremost threats to nearshore ecosystems worldwide, yet there is generally a paucity of data on the assimilation of terrestrial nutrient inputs by marine organisms. Moreover, there have been few studies that have effectively developed bioindicators as a tool for watershed management. At a national level, New Zealand has a recent history of rapid ecosystem change associated with changing land use practices (Parliamentary Commissioner for the Environment 2004), yet there are no studies that have related these changes in the catchment to terrestrial nutrient inputs in estuarine and coastal habitats and the potential impacts on the structure and function of these nearshore ecosystems.

This study attempted to address this knowledge gap by examining the effect of different land use practices on the assimilation of terrestrial nutrient sources by marine organisms.

1.2 Objectives

Specifically, this project aimed to

(i) investigate linkages between land use patterns in catchments and nitrogen loading to recipient estuaries and coastal ecosystems;

(ii) characterise isotopic signatures of selected bioindicator organisms in relation to different terrestrial nutrient loads; and

(iii) validate the use of bioindicators using controlled laboratory and field experiments.

The outcomes of the research provide quantitative data on the applicability of different indicators to detect and quantify changes in anthropogenic nitrogen inputs to coastal ecosystems. In addition, the project provides novel information on the ecology of New Zealand's nearshore environment, which can facilitate the development of an ecosystem-based approach to management of New Zealand estuarine and coastal environments.

1.3 Bioindicators as proxies of ecosystem health

Estuarine bioindicators are species, populations, or communities that can be used to assess the effects of a stimulus on an ecosystem. By proxy, they represent the cumulative effects of exposure to ecosystem-wide disturbance (Adams 2005). In contrast to biomarkers (which indicate exposure, not effects), bioindicators usually have low sensitivity and little relationship to the stressor(s), but reflect a relatively long duration of response and are more ecologically relevant (Adams 2005). Moreover, since they represent an integration of multiple stressors, they are particularly useful in coastal and estuarine ecosystems that experience rapid fluctuations in physicochemical parameters across multiple spatial and temporal scales. Consequently, bioindicators have been developed at all trophic levels to quantify a range of organism responses (Wilson 1994). Typical bioindicators that have been used to assess water quality and ecosystem function include bacterial and phytoplankton community composition (Paerl et al. 2003a, 2003b), and stable isotope ratios of macroalgae (Costanzo et al. 2001, Savage & Elmgren 2004), bivalves (Fry & Allen 2003), benthic deposit-feeders (Van Dover et al. 1992), and fish (Hansson et al. 1997).

1.4 Stable isotopes as tracers of nutrient and organic matter sources

Stable isotope ratios in organisms can provide information on the origin and assimilation of different organic matter sources in coastal food webs (Lajtha & Michener 1994, Fry 2006 and references therein). In particular, stable carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) isotope ratios can be used to elucidate the influence of anthropogenic nutrient enrichment in estuarine systems (Bannon & Roman 2008) and to trace terrestrial organic matter sources as they move through food webs (McLeod & Wing 2007). Moreover, stable isotopes integrate data across spatial and temporal scales that are appropriate to food web dynamics (Vander Zanden & Rasmussen 1999, Gartner et al. 2002), which makes them a convenient tool and informative means by which the importance of terrestrial inputs in structuring higher trophic levels can be assessed. Thus, the approach can provide valuable insights into the effects of different land-derived sources of nutrient loading and the movement of nutrients through food webs.

This study used stable isotope ratios to assess the effects and assimilation of different land-derived nutrient sources on recipient coastal ecosystems. In addition, I combine isotopic signatures with conventional gut content analyses and biological measures (including growth responses, condition indices) to assess ecological effects of different nutrient loading regimes on recipient coastal ecosystems.

2. METHODS

2.1 Study sites and description of catchments

Estuaries and coastal inlets were selected to encompass a range of different land use practices. Six study areas in the South Island were selected to represent coastal habitats influenced by (a) predominantly agricultural inputs (Waikouaiti estuary, Tokomairiro estuary), (b) urbanised inputs (Otago Harbour, Bluff Harbour), and (c) the persistence of native forested catchments (Paterson Inlet – Stewart Island, Doubtful Sound – Fiordland) (Figure 1).

Catchments and subcatchments for each study area were delineated at a 1:50 000 mapping scale using the River Environment Classification (REC), the geographical information systems (GIS) classification of New Zealand rivers developed by NIWA. Land use in the catchments of the study

areas was differentiated into 16 land-cover categories (Figure 2-4) according to the LINZ Land Cover Database 2 (LCDB2) classification. The LCDB2 is based on Landsat 7-ETM+ (15 m spatial resolution) and SPOT 4 (20 m spatial resolution) imagery taken between October 2001 and March 2002, which has a 1 ha minimum mapping unit (MMU). Area estimates (in hectares and relative percent cover) of each land use type were calculated using the GIS mapping software ArcGIS v. 9.2 (ESRI).

2.2 Nitrogen loading model

The Nitrogen Loading Model (NLM) developed to quantify land-derived nitrogen loads to receiving waters (Valiela et al. 1997) was used as an initial model to estimate total nitrogen loading. The NLM estimates total nitrogen from atmospheric and land-derived sources and tracks these various sources as they traverse soils and travel in groundwater to receiving estuaries (Valiela et al. 2004). However, the NLM was developed to predict total dissolved nitrogen loads to shallow estuaries from rural suburban watersheds in USA dominated by septic tank wastewater inputs. Since septic tanks are not a dominant nutrient source in New Zealand, the model was modified and New Zealand values used to estimate nitrogen inputs to the study estuaries and coastal systems. In particular, atmospheric deposition, fertiliser use, residential wastewater, and biological nitrogen fixation were estimated for catchments and selected subcatchments based on the GIS land use categories quantified in Section 2.1 and the respective nitrogen inputs to receiving estuaries calculated. Livestock fed on locally grown pasture are not treated as new sources of nutrients as the nitrogen in their waste products derives from nitrogen in the atmosphere and fertiliser applied to that parcel of land. Thus, the model already accounts for the nitrogen passing through the livestock (Valiela et al. 1997). The model input components and complex losses that occur on the catchment surfaces, in freshwater bodies, and in groundwater to emerge at the seepage face on the estuary shore are listed in Table 1.

The modified NLM was run under two scenarios: using (i) individual site-specific catchments, and (ii) multiple subcatchments, where all subcatchments upstream of two sampling sites were included in the model calculations. Comparison of the NLM output between the two applications showed that while there was some variance in nitrogen loads for individual sites, the total nitrogen loading rates for each estuary were consistent when normalised to watershed area. Land-derived nitrogen loads for Waikouaiti and Stewart Island sites were consistent between the two scenarios, while the model performed better for Otago Harbour when multiple subcatchments were examined. Moreover, since water residency time in the inner harbour is in the order of days, it is more applicable to assess nitrogen loads using all subcatchments upstream of the sampling site. Consequently, the data reported below are for nitrogen loads using multiple subcatchments.

2.3 Field sampling

At each study area, sites were selected to encompass a salinity gradient from brackish water to fully marine environments. On average, five sites per estuary/inlet were chosen, and at each sampling site, water samples and replicate samples of selected organisms were collected. Sampling was conducted in spring (November 2005), summer (January-February 2006, January 2007), autumn (May 2006, May 2007), and winter (August-September 2006) to capture seasonal shifts in bioindicators.

Water samples were collected in triplicate from each site, filtered (0.45 μm) and frozen at -20 ºC until analysis within two months of collection. Dissolved nutrient concentrations (NH_4^+, NO_2^-/NO_3^- and when possible PO_4^-) were determined on an autoanalyser at the Botany Department, University of Otago. From January 2007, suspended particulate organic matter (SPOM) samples were also taken at

each site (this was in addition to the proposed sampling methods outlined in the contract). Water was filtered (at least 2 litres per site) and the particulate organic matter (over 0.45 µm) retained on filters stored frozen at -20 ºC until analysis for isotopic ratios at Isotrace Limited, Dunedin.

Organisms were selected that (i) had a cosmopolitan distribution across New Zealand estuaries and coastal ecosystems and (ii) represented different trophic levels. Consequently, selected potential bioindicator species include: (a) the chlorophyte macroalga, *Ulva* spp. (sea lettuce), as a primary producer, (b) the filter-feeding bivalves, *Austrovenus stutchburyi* (New Zealand Littleneck clam) and *Mytilus galloprovincialis* (Mediterranean mussel), as primary consumers, and (c) fishes including the labrid, *Notolabrus celidotus* (spotty) and *Grahamina nigripenne* (estuarine triplefin). In order to reconstruct food webs for the estuarine ecosystems, the regular suite of bioindicator species was complemented with additional species including seagrass, microphytobenthos, amphipods, etc. All organisms were frozen at -20 ºC until analysis.

2.4 Stable isotope preparation and analysis

Organisms were defrosted, rinsed in distilled water and selected tissues dissected for analysis. Tissues selected for analysis include the outer growth margin or growth tips in macroalgae (*Ulva* spp., *Gracilaria* spp.) and seagrass, the foot muscle tissue (and gills in selected samples) in *Austrovenus stutchburyi*, abductor muscle (gills and foot muscle in some samples) in *Mytilus galloprovincialis*, and the dorsal muscle tissue in fishes, *Notolabrus celidotus* and *Grahamina nigripenne*. Tissues were dried at 60 ºC for at least 72 hours, and ground and homogenised. Aliquots (about 1 mg) were weighed into tin capsules and analysed on a Carlo rRba NA1500 elemental analyser linked to a Sercon Hydra 20/20 stable isotope ratio mass spectrometer at Isotrace Limited. Stable isotope ratios are reported as the ratio between the heavy to light isotope ratio (^{15}N:^{14}N, ^{13}C:^{12}C) in parts per thousand deviation (‰) from laboratory calibrated standards.

Since coastal ecosystems are generally nitrogen limited (Howarth 1988), and catchments in the study region have undergone shifts in nitrogen flux (from increased fertiliser use particularly urea and livestock numbers), the focus of this report is on stable nitrogen isotope values ($\delta^{15}N$) in selected organisms. However, where relevant, and for partitioning sources of organic matter, dual isotopes ($\delta^{15}N$, $\delta^{13}C$) are reported.

2.5 Partitioning organic matter sources in biota

The percentage contribution of different organic matter sources to higher trophic levels in the respective coastal food webs was estimated using isotopic mixing models. Specifically, dual isotope signatures ($\delta^{15}N$, $\delta^{13}C$) of fish were used to resolve and partition three contributing nutrient sources using the following equation:

$$\delta_{organism} = f_A\delta_A + f_B\delta_B + f_C\delta_C$$

where $\delta_{organism}$ is the isotopic ratio of the organism, δ_A is the isotopic ratio of source (end-member) A, δ_B is the isotopic ratio of source B, δ_C is the isotopic ratio of source C, and f is the mean proportion of each of the sources in the organism (Phillips & Gregg 2001).

The stable isotopic ratios and elemental composition of potential bioindicators were tested across sites within each study site to discern across-estuarine gradients in bioindicators, and among estuaries to discern site-specific differences in stable isotope ratios. Data were tested for normality and homogeneous variances. If the data weren't normally distributed, they were log-transformed. Data that violated the assumptions of normality were analysed using non-parametric tests (Kruskal-Wallis).

Data that were normal were analysed using ANOVA. Data that were normally distributed but didn't have equal variances were analysed using Welch ANOVA. Regressions were performed between the stable isotope ratios of selected organisms and measured nutrient concentrations and modelled nitrogen loads to assess how the bioindicators respond to gradients of nutrient availability.

2.6 Fish growth curves

The pair of sagittal otoliths was removed from spotties and triplefins and only otoliths from the right side of the fish used for age estimates as morphological differences can occur between otoliths of a pair. Full methods of otolith preparation and validation of the counts were outlined by Hammond (2006). Growth data were fitted to the von Bertalanffy growth function by the least-squares method using Excel software. This equation has been used widely in ecology and fisheries and describes the average population growth trajectory when data are fitted to it using least-squared residuals (Haddon 2001). Age-at-length data for both spotty and triplefins were fitted to the von Bertalanffy equation, as follows:

$$L_t = L_\infty \ (1 - e^{-k\,[\,t - t0\,]})$$

where L_t is length at age t, L_∞ refers to the asymptotic average maximum body size, k is a growth rate coefficient describing rate at which maximum size is reached, and t_0 is the hypothetical fish size at age zero. t_0 is extrapolated from data and is also difficult to interpret in an ecologically meaningful sense (Haddon 2001), so it was constrained to zero for all growth curves.

Growth rates were compared between estuaries using Analysis of Residual Sum of Squares (ARSS). This method compares non-linear curves of the same type with the null hypothesis that they are equivalent descriptions of the data (Haddon 2001). Growth curves were compared in a pair-wise fashion to identify where differences existed (at $\alpha = 0.05$ significance level). Visual comparisons, with reference to L_∞ and k, assessed how curves differed and to quantify whether growth rates were greater or less between estuaries. To increase sample size and complement fish growth estimates, fish were also collected from Catlins and Tautuku river estuaries.

Stomach contents (whole digestive tracts) were analysed from *Notolabrus celidotus* and *Grahamina nigripenne* from the different coastal areas and diet was quantified using volumetric and numeric prey counts. The Index of Relative Importance (IRI) is calculated as:

$$IRI = (\%V + \%N) \times \%FO$$

where %V is the percentage of a prey category volume relative to total volume of pooled stomachs for a population, %N is the same proportion for numeric prey counts, and %FO is the proportion of stomachs in the population containing that prey category (Cortes 1997).

3. RESULTS AND DISCUSSION

3.1 Land use and nutrient flux

Geographical information systems (GIS) data verified the initial hypothesis that the six study sites reflect different relative percentage cover of land use types from primarily pastoral lands to pristine indigenous forest and shrubland areas (Figure 2-4). The catchments of study sites chosen as representative pristine coastal ecosystems were dominated (over 90%) by indigenous forest and shrub. In contrast, nearshore environments influenced by pastoral land use in the catchments, had 62% to

83% agricultural land use, and urban influenced coastal systems were bordered by impervious surfaces (urban cover) and areas of indigenous shrubland and pastoral land use. These land use types were used to estimate total nitrogen loads at an ecosystem level and correlated against water column nutrient concentrations and nutrient proxies (e.g., mean $\delta^{15}N$ values of macroalgae for each coastal system; see below and Figure 6).

Three study areas (Otago Harbour, Stewart Island, Waikouaiti) were influenced by multiple catchments and potential nutrient source pools (Figure 5). These areas were therefore also analysed at a subcatchment level and nitrogen loading rates estimated for individual sites (Table 2; see Methods for description of multiple upstream subcatchments). These data were used to assess gradients in nitrogen loading rates across individual coastal ecosystems and correlated with site-specific bioindicators.

Nitrogen loading models based on the GIS land use data for subcatchments demonstrated that nitrogen inputs to coastal ecosystems increased directly with the fraction of land area in agriculture (R^2 = 0.99, Figure 6). The model showed that as agricultural practices intensified, the resulting nitrogen loads to recipient water bodies increased and the percent contribution from fertiliser increased. The different estuaries were influenced by various nutrient source pools and exhibited a wide range of nitrogen loads from 26 to 576 791 kg yr^{-1} (Table 2). Land-derived nitrogen loading rates normalised to catchment area ranged from 0.6 (for Stewart Island) to 17 kg ha^{-1} yr^{-1} (for Waikouaiti). These values are similar to the annual total nitrogen inputs estimated for the entire Otago region, which are 26 kg ha^{-1} yr^{-1} (Parfitt et al. 2006). The nitrogen loads for the study sites in Otago were within the range of lower values reported for watersheds in northeast USA, where nitrogen loads were estimated as between 25-199 kg ha^{-1} yr^{-1} for Pleasant Bay, Cape Cod (Carmichael et al. 2004), and 14–601 kg ha^{-1} yr^{-1} for eight other water bodies in Cape Cod, MA (Carmichael & Valiela 2005). These relatively lower values for New Zealand compared to USA estuaries are likely due to lower population density, fewer urban wastewater inputs, and less cropping in New Zealand watersheds that were included in the present study. Nevertheless, the values are similar to regional nitrogen budgets (Parfitt et al. 2006) and support the use of this approach to estimate nitrogen loads to recipient coastal water bodies in New Zealand.

Moreover, environmental conditions including nitrate/nitrite, ammonia, phosphate, dissolved oxygen, and chlorophyll-*a* concentrations, varied along a continuum consistent with catchment land use differences (Hammond 2006). Water column dissolved inorganic nitrogen (DIN) concentrations among sites generally reflected the modelled nitrogen loads; however, due to the high temporal and spatial variation in nutrient concentrations and the integrated nature of the nutrient proxies (stable isotope ratios in biota), I focus on the output of the nitrogen loading models for each catchment and subcatchment.

3.2 Bioindicators

3.2.1 Isotopic gradients within individual coastal ecosystems (local scale)

Stable nitrogen and carbon isotope ratios and elemental composition (molar C:N) of primary producers (e.g., *Ulva* spp.) differed significantly across individual coastal systems. Macroalgal $\delta^{15}N$ signatures generally increased from the inner brackish water sites to the outer marine sites, with peak $\delta^{15}N$ values near the mouth of the estuary (near the estuarine mixing zone region) (Figure 7a). This across-estuarine gradient in isotopic values varied seasonally and was most pronounced during the spring-summer season, which coincides with the main algal growth season.

These spatial gradients in macroalgae are in accordance with other studies that have recorded similar patterns in summer in stable isotope ratios and nitrogen content of primary producers across individual coastal embayments (e.g., Fourqurean et al. 1997). The patterns in macroalgal nitrogen content provide information on the spatial and temporal variation in nutrient availability in the different coastal systems. The variation in macroalgal $\delta^{15}N$ values reflects DIN concentrations in the water column and indicates the importance of *in situ* denitrification in the nitrogen cycle in these shallow water systems. Denitrification is a bacterially mediated process that is normally amplified during the summer months (Seitzinger et al. 2006), and thus is likely responsible for the progressive increase in $\delta^{15}N$ values along the freshwater–marine continuum that is most pronounced during warmer months. Denitrification causes ^{15}N enrichment of the water column dissolved inorganic nitrogen (DIN) pool as nutrients are processed along the estuary (Heaton 1986), which in turn is reflected in the macroalgal tissues that assimilate the progressively ^{15}N-enriched ammonia and nitrate source signature. Thus, the analysis of patterns in variation of elemental content and isotopic composition of macroalgae on a local scale is a powerful tool for investigating ecosystem-scale processes in coastal marine systems.

Stable nitrogen and carbon isotope ratios of primary consumers (e.g., *Austrovenus stutchburyi)* also showed variation across individual coastal ecosystems. In contrast to $\delta^{15}N$ values for macroalgae, however, $\delta^{15}N$ values of cockles were more enriched in the inner, more freshwater sites and generally decreased towards the mouth in nutrient enriched coastal systems (Figure 7b). At pristine sites (Stewart Island), there was no significant directional change in $\delta^{15}N$ values of cockles; however there was a general decrease in $\delta^{13}C$ values along the freshwater to marine continuum (Heggie 2008). The patterns in isotopic ratios for primary consumers were, however, less pronounced than for macroalgae, which is largely due to filter feeders assimilating particulates from a broader area of the coastal habitat and integrating the $\delta^{15}N$ over longer timescales (McKinney et al. 2002, Fry & Allen 2003).

The results of the study thus provide further evidence for using multiple complementary bioindicators to assess ecosystem health (Wilson 1994, Adams 2005). The spatial and temporal gradients in chemical markers for primary producers reflected variance in nutrient availability and ecosystem-scale processing of nutrients, while the primary consumers reflected a more integrated picture of the particulate organic matter sources across different coastal systems.

3.2.2 Differences in isotopic values among coastal ecosystems (regional scale)

The mean $\delta^{15}N$, $\delta^{13}C$, and molar C:N values of primary producers (e.g., *Ulva* spp.) differed significantly among different estuarine and coastal ecosystems. Post-hoc tests showed that the groupings of study areas that were significantly different from one another shifted seasonally, but some consistent trends emerged. When the data for $\delta^{15}N_{Ulva}$ are combined across all sites (01–05) and for all seasons, macroalgae are enriched ($\delta^{15}N$ = 9‰) in areas influenced by urban watersheds and agriculture–dominated systems ($\delta^{15}N$ = 7.5‰) relative to pristine coastal environments ($\delta^{15}N$ = 6.5‰) (Figure 8). However, when the data are analysed on a seasonal basis, this distinction among locations varies. It is noteworthy that there is no significant difference between urban and pastoral areas during winter and the pristine coastal areas exhibit relatively heavy $\delta^{15}N$ values, possibly due to upwelling of isotopically heavy bottom-water nitrate off the coast which is advected into these coastal areas (Voss et al. 1997, Altabet 2001). In contrast, the variance in $\delta^{15}N$ between urban, agricultural, and forested areas is most pronounced during summer (Figure 8), which coincides with the maximum growth period for algae. The results therefore suggest that sampling macroalgae to detect regional differences in isotopic values should be conducted in summer.

While patterns of isotopic values for primary producers showed variance among study locations, trends in $\delta^{15}N$, $\delta^{13}C$, and molar C:N values for primary consumers did not reflect clear differences between nutrient enriched and pristine areas (Heggie 2008). The mean $\delta^{15}N$ values for cockles (*Austrovenus stutchburyi*) ranged between 8.6 and 10.5‰ for all seasons combined, with no directional pattern in relation to land use in surrounding catchments. There was also less temporal variance in isotopic values for cockles, which was explored in isotopic turnover experiments (Heggie and Savage, *in preparation*). Therefore, in contrast to several studies that have used filter-feeding bivalves as indicators of watershed-derived nutrient inputs to aquatic bodies (McKinney et al. 2002, Fry & Allen 2003, Anderson & Cabana 2005), I found no clear distinction in isotopic values among coastal ecosystems when all sites from individual estuaries were combined.

3.2.3 Relationships between bioindicators and nutrient loads

In order to test the value of using isotope ratios in biota as proxies of terrestrial nutrient inputs, the isotopic values were correlated with measured water column nutrient concentrations and modelled nitrogen loads at local and regional levels.

The mean $\delta^{15}N$ value of *Ulva* for all study sites (local scale) and all seasons showed a positive but weak relationship ($R^2 = 0.15$) with total dissolved inorganic nitrogen (DIN) concentration (Figure 9). However, when $\delta^{15}N_{Ulva}$ was correlated with DIN during the growth season (spring–summer) within an individual coastal system, this relationship was generally much stronger. For example, the regression between $\delta^{15}N_{Ulva}$ and DIN concentration – both collected in spring – in Otago Harbour shows that water column nitrogen explains 86% of the variance in stable nitrogen isotope ratios in macroalgae.

At a regional scale, when mean $\delta^{15}N$ values (averaged across all sites) of primary producers (*Ulva* spp.) are regressed against nitrogen flux estimates for the respective coastal ecosystems (estimated from the nitrogen loading model), there was a positive relationship ($R^2 = 0.35$) between $\delta^{15}N$ and total nitrogen loads (Figure 10a). The relationship between $\delta^{15}N_{Ulva}$ was more strongly related to wastewater (Figure 10b) and fertiliser nitrogen source pools than total N loads, particularly during the spring and summer algal growth period (Table 3).

In conclusion, macroalgal $\delta^{15}N$ values increase significantly with percent wastewater N loading and DIN concentration. This is in agreement with other studies that have used macrophyte $\delta^{15}N$ values to identify relative inputs of land-derived wastewater N (McClelland & Valiela 1998, Gartner et al. 2002, Cole et al. 2004, Savage & Elmgren 2004), and is because wastewater $\delta^{15}N$ values in groundwater are more enriched than fertiliser or atmospheric N sources (Heaton 1986). Analysing macroalgal tissues for $\delta^{15}N$ values thus provides an effective and simple tool to assess the effects of watershed urbanisation on coastal water bodies. The findings of this study support analysing *Ulva* spp. samples during the algal growth period in spring-summer to best reflect local wastewater N loads. Further, sampling of stable isotope ratios in organisms as bioindicators needs to be conducted using spatially explicit sampling schemes due to the isotopic gradients across individual coastal ecosystems, particularly during the summer months.

3.3 Ecological effects of nutrient enrichment in coastal ecosystems

3.3.1 Growth of secondary consumers, *Notolabrus celidotus* (spotties) and *Grahamina nigripenne* (estuarine triplefins), among sites

Spotties (*Notolabrus celidotus)* sampled from the different coastal ecosystems ranged from 1 to 12 years in age and 44–333 mm in length. Von Bertalanffy growth curves were fitted to sites with sufficient sample size and showed significant differences in age-at-length (L_∞) values and growth rate coefficients (k), the rate at which maximum size is reached (Table 4). Growth rate curves showed that spotty samples from Waikouaiti estuary and Doubtful Sound shared similar trajectories (Figure 11). Similarly, the Freshwater (Stewart Island) and Catlins estuaries had similar forms, although fish from Stewart Island were consistently smaller at comparable ages. ANOVA tests showed that at age 3, Stewart Island spotties had the lowest mean length and differed significantly from the longest fish collected from the Catlins estuary ($F=18.22$, $p=0.000$). By age 5, however, Waikouaiti spotties had the greatest mean length, which differed significantly from the shortest Stewart Island fish ($F=4.04$, $p=0.028$). Differences in growth and muscle lipid content of spotties among estuaries were best explained by variation in primary production (measured as chl-*a* concentration).

Estuarine triplefin (*Grahamina nigripenne*) growth data included fish ranging in age from 1 to 7 increments and 47–100 mm in length. Growth curve comparisons showed significant differences in L_∞ between the northern, more nutrient-enriched areas (Waikouaiti and Tokomairiro) and southern, more pristine sites (Tautuku and Stewart Island). Thus, similar to spotties, estuarine triplefin growth appeared to be enhanced in nutrient enriched estuaries, but this trend was inseparable from temperature effects.

Growth rate patterns differed between spotties and triplefins. Both appeared to exhibit slow growth at Stewart Island, although spotties reached a larger relative size. L_∞ was highest in the more nutrient enriched northern estuaries for triplefins, however it was among the lowest for spotties. Comparisons of growth rates, reproductive traits (GSI, gonad somatic index) and condition indices (lipid content) between spotties and triplefins therefore reflect environmental conditions in divergent ways, possibly due to differences in habitat use. This was further supported by the gut content and stable isotopic analyses.

3.3.2 Differences in diets of secondary consumers among sites

Analysis of 254 stomachs from *Grahamina nigripenne* and 138 stomachs from *Notolabrus celidotus* revealed some important differences among the different study areas. In particular, prey richness appeared far greater at Stewart Island than at the other coastal areas studied (Table 5). Shannon-Weiner diversity (H') and Pielou's evenness index (J') confirmed that fish from Stewart Island consumed prey more evenly across more prey taxa than fish at the other coastal areas. Tokomairiro triplefins had particularly uneven diets, feeding almost exclusively on amphipods.

Benthic prey, especially amphipods, dominated triplefin diets in all coastal ecosystems (Table 6). Despite some differences in prey importance among the different sites, estimates of trophic level from stomach contents (Cortes 1999) were remarkably uniform, averaging 3.01 ± 0.02 between sites. Amphipods were also an important dietary item for spotties collected at Toko mouth, but were second in importance to brachyurans for spotties at all the coastal areas that were well sampled (Table 7). As with triplefins, Stewart Island spotties consumed considerably more prey items than other populations.

Fish muscle $\delta^{15}N$ and $\delta^{13}C$ values supported the gut content analyses and refined the importance of different local carbon and nitrogen dietary sources. ^{15}N enrichment of lower trophic benthic food web components (e.g., mudsnails, amphipods) and triplefins in the nutrient-enriched Toko mouth estuary confirmed that the input of ^{15}N-enriched wastewater and manure is assimilated by local food webs (Figure 12).

3.3.3 Differences in food web structure and function among sites

A food web model based on dual isotope ratios was developed using (a) data pooled from all the study sites in southern New Zealand to develop a generalised food web model for southern New Zealand estuaries and (b) for each coastal ecosystem to compare potential differences among ecosystems. The generalised food web showed that triplefins were, on average, more depleted in $\delta^{15}N$ values than spotties (Figure 13). This relationship parallels trophic level estimates derived from stomach contents. Both species were differentially ^{15}N-enriched above primary consumers, but were similar in $\delta^{13}C$ values, suggesting similar carbon sources. Primary consumers, in turn, were supported by a mixture of ^{13}C-depleted carbon sources such as microphytobenthos (MPB), suspended particulate organic matter (SPOM) and macroalgae, and more ^{13}C-enriched sources like seagrass (*Zostera novazelandica*). Filter-feeding bivalves (e.g., cockles, *Austrovenus stutchburyi*) were more reliant on ^{13}C-depleted sources like SPOM and MPB, while amphipods appeared more reliant on seagrass carbon sources.

Two-source isotopic mixing models (Phillips & Gregg 2001, 2003) were used to assess differences in food web structure and the relative importance of the different organic matter sources supporting food webs among the different coastal ecosystems. These models suggested that for the generalised food web (pooled data from all study areas), both fish species were predominantly (over 50%) supported by macroalgal production, while seagrass contributed moderately (7–33%) and SPOM made only minor contributions (0–20%). However, when individual food webs were compared, differences in food web support among estuaries hinted at alterations in carbon and nitrogen routing due to nutrient enhancement. While not conclusive, gut content and stable isotope analyses implied the importance of seagrass production in pristine coastal ecosystems, and SPOM (composed largely of phytoplankton) or microphytobenthos in nutrient-enriched areas (Figure 14).

4. CONCLUSIONS AND RECOMMENDATIONS

- The six coastal study sites in Otago and Southland were influenced by different nutrient source pools and varying nitrogen loads (0.6–17 kg ha^{-1} yr^{-1}), consistent with different land use practices in their respective catchments.

- Nitrogen loading models suggest that nitrogen inputs to nearshore environments increased directly with intensified agricultural practices in catchment. Thus integrated management needs to minimise run-off from watersheds, in particular from agriculture, to obtain good ecological status of coastal ecosystems in New Zealand.

- Stable nitrogen isotope ratios ($\delta^{15}N$) in organisms are useful bioindicators that can help managers detect changes in nitrogen inputs to coastal and estuarine ecosystems. The $\delta^{15}N$ values of primary producers and primary consumers reflect water column dissolved inorganic nitrogen (DIN) and particulate nitrogen sources, respectively. Moreover, various organisms experience different turnover rates and thus reflect nutrient conditions over different timescales. These results support the use of multiple complementary bioindicators to assess ecosystem health.

- The $\delta^{15}N$ value of *Ulva* spp. was positively related to wastewater nitrogen, in particular during the algal growth period (spring-summer). Thus analysing *Ulva* spp. samples in summer is an effective way to assess the influence of human sewage inputs in coastal water bodies.

- Isotopic gradients in primary producers and consumers varied locally and seasonally, reflecting estuarine gradients in environmental variables. This highlights the importance of sampling over highly resolved spatial and temporal scales.

- Differences in nutrient loads among estuaries were transmitted up the food web with differences in growth rates and diets recorded in fishes. For example, spotties (*Notolabrus celidotus*) exhibited differences in growth and muscle lipid content among estuaries that was best correlated with differences in primary production (chl-*a* concentration). Growth was also enhanced in estuarine triplefins (*Grahamina nigripenne*) in nutrient enriched estuaries, however the trend could not be separated from temperature effects. Therefore enhanced nutrient levels may increase food availability and have positive effects on growth of secondary consumers including fishes in estuaries.

- Nutrient-enriched coastal ecosystems may support lower species diversity of ecologically important species. Fishes (*Notolabrus celidotus*; *Grahamina nigripenne*) consumed considerably more diverse prey items in pristine coastal areas. In addition, food web models showed a shift in the relative importance of different organic matter sources supporting coastal food webs in nutrient-enriched estuaries. Consequently, elevated nutrient levels in coastal and estuarine ecosystems can diminish biodiversity and cause shifts in food web structure.

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Table 1: The input parameters and loss functions used in the Nitrogen Loading Model (NLM) adapted for New Zealand estuaries and coastal environments. Note that biological nitrogen fixation (BNF) by invasive gorse and broom has been included in the model and agricultural practices have been modified for the New Zealand setting.

Nitrogen to and through watershed surfaces (kg yr^{-1})
via atmospheric deposition to:

Natural vegetation [1]: atmospheric deposition (kg•ha^{-1}•yr^{-1}) x area (ha) of naturally vegetated land x 35% not retained in plants and soil

Turf [2]: atmospheric deposition (kg•ha^{-1}•yr^{-1}) x area (ha) of turf x 38% not retained in plants and soil

Horticultural land [3]: atmospheric deposition (kg•ha^{-1}•yr^{-1}) x area (ha) of horticultural land x 38% not retained in plants and soil

Impervious surfaces [4]: atmospheric deposition (kg•ha^{-1}•yr^{-1}) x [area (ha) of roads + commercial areas]

Ponds and Lakes [5]: atmospheric deposition (kg•ha^{-1}•yr^{-1}) x area (ha) of ponds and lakes

Wetlands [6]:atmospheric deposition (kg•ha^{-1}•yr^{-1}) x area (ha) of wetlands

via fertiliser application to:

Agricultural land [7]: [crop fertilisation rate x area (ha) under cultivation x 68% not lost as gases] - nitrogen removed as crop †

via biological nitrogen fixation to:

Pasture legumes [8]: fixation by pasture legumes (kg•ha^{-1}•yr^{-1}) x area (ha) of pasture legumes x 38% not retained in plants and soil

Gorse and broom [9]: fixation by gorse and broom (kg•ha^{-1}•yr^{-1}) x area (ha) of pasture legumes x 35% not retained in plants and soil

Native forest lichen [10]: fixation by native forest lichen (kg•ha^{-1}•yr^{-1}) x area (ha) of pasture legumes x 35% not retained in plants and soil

Nitrogen to and through vadose zone and aquifer (kg yr^{-1}):

Via nitrogen percolating diffusely from watershed surface [11]: [sum of 1-4 + 7-10] x 39% not lost in vadose x 65% not lost in aquifer

Via nitrogen percolating diffusely through ponds and lakes [12]: [5] x 44% not lost in vadose x 65% not lost in aquifer

Via nitrogen percolating diffusely through wetlands [13]: [6] x 22% not lost in vadose

Via wastewater from wastewater treatment plants [14]:

Nitrogen loading to estuary (kg yr^{-1}): sum of items 11 - 14

† nitrogen removed as crop = [export - import]

Table 2: Watershed area and percent agriculture in the mutiple subcatchments above sites in a pristine estuary (Stewart Island: SI01-SI05), urban coastal inlet (Otago Harbour: OH01-OH05) and a predominantly agricultural estuary (Waikouaiti: WK01-WK05). The calculated nitrogen (N) loading rates for each site are presented as total N load (kg yr^{-1}), percent fertiliser contribution, and normalised to the watershed area (kg ha^{-1} yr^{-1}) for each site.

	Watershed		Calculated Nitrogen Loads		
Site	Watershed Area (ha)	Percent Agriculture	Total N Load (kg N yr^{-1})	Percent Fertilizer	Total N Load (kg N ha^{-1} yr^{-1})
SI04	47.3	0.0	26.2	0.0	0.6
SI02	54.6	0.0	34.0	0.0	0.6
SI05	43.9	0.0	27.4	0.0	0.6
SI03	409.5	0.0	258.2	0.0	0.6
SI01	31792.9	0.0	20047.8	0.0	0.6
OH01	5694.9	26.7	32883.6	67.8	5.8
OH02	391.6	50.9	3892.8	75.0	9.9
OH04	1276.0	57.9	14210.9	76.1	11.1
OH03	2528.6	58.3	28394.6	76.1	11.2
WK02	228.6	65.1	2834.0	76.8	12.4
OH05	662.0	70.7	8912.8	76.9	13.5
WK01	39797.7	77.1	576790.8	78.0	14.5
WK04	396.1	91.2	6743.9	78.5	17.0

Table 3: Relationship between macroalgal (*Ulva* spp.) $\delta^{15}N$ value and different nitrogen source pools: A) wastewater N inputs and (B) fertiliser inputs. Nitrogen inputs are based on NLM estimates for each coastal site. Regressions were performed for all seasons combined and individual seasons.

RELATIONSHIP BETWEEN $\delta^{15}N_{ULVA}$ AND:

A) LOG WASTEWATER N LOAD

	R^2 value
All seasons	0.78
Spring	0.84
Summer	0.89
Winter	0.15
Autumn	0.41

B) LOG FERTILISER N LOAD

All seasons	0.34
Spring	0.75
Summer	0.70
Winter	0.31
Autumn	0.01

Table 4: Von Bertalanffy parameters for *Notolabrus celidotus* growth curves from four coastal ecosystems, where L_∞ = age-at-length (see methods for equation), k = the growth rate coefficient describing rate at which maximum size is reached, and n = number of samples analysed.

STUDY SITE	L_∞	k	n
Waikouaiti	217.83	0.51	33
Catlins	325.90	0.24	49
Stewart Island	363.27	0.13	45
Doubtful Sound	207.05	0.48	27

Table 5: Prey taxa richness, Shannon-Weiner diversity indices (H') and Pielou's diversity indices (J') for stomach contents of (A) estuarine triplefins, *Grahamina nigripenne*, and (B) spotties, *Notolabrus celidotus*, collected from different coastal ecosystems. *, insufficient samples to calculate indices.

	WAIKOUAITI	TOKO MOUTH	CATLINS	TAUTUKU	STEWART ISLAND
(A) *G. nigripenne*					
Total prey taxa	7	6	8	9	15
Diet diversity (H')	2.17	2.17	2.17	1.95	3.04
Diet evenness (J')	0.19	0.06	0.12	0.17	0.38
(B) *N. celidotus*					
Total prey taxa	13	*	11	*	17
Diet diversity (H')	1.08	*	1.56	*	1.22
Diet evenness (J')	0.42	*	0.65	*	0.42

Table 6: Diet composition from stomach contents of *Grahamina nigripenne* from five study areas. Diets are presented as the percent Index of Relative Importance (IRI). Prey taxa categories typically represent class or order. n, sample size at each site.

PREY TAXON	WAIKOUAITI (n=50)	TOKO MOUTH (n=54)	CATLINS (n=57)	TAUTUKU (n=43)	STEWART ISLAND (n=42)
Amphipoda	89.9	97.3	93.8	92.5	75.4
Brachyura	0.1	-	1.4	2.3	9.2
Algae	2.1	0.1	3.7	0.6	2.9
Isopoda	0.7	0.6	1	1.5	3.5
Pycnogonida	6.4	-	-	-	-
Crustacea	0.5	0.3	-	1.7	2.3
Balanomorpha	-	-	-	-	0.5
Bivalvia	0.3	-	-	1.2	0.2
Polychaeta	-	1.5	-	-	-
Teleostei	-	-	-	-	1.4
Archaegastropoda	-	-	-	0.1	0.9
Mesogastropoda	-	-	-	-	0.6
Nemertea	-	-	-	-	0.5
Diptera	-	-	-	-	0.3
Polyplacophora	-	-	-	-	0.1
Decapoda	-	<0.1	-	-	<0.1
Angiosperm	-	-	<0.1	-	<0.1
Rhodaphyta	-	-	-	<0.1	-
Insecta larvae	-	-	-	<0.1	-
Euphausida	-	-	<0.1	-	-
Paricarida	-	-	<0.1	-	-
Dermaptera	-	-	<0.1	-	-

Table 7: Diet composition from stomach contents of *Notolabrus celidotus* from five study areas. Diets are presented as the percent Index of Relative Importance (IRI). Prey taxa categories typically represent class or order. n, sample size at each site.

PREY TAXON	WAIKOUAITI (n=31)	TOKO MOUTH (n=7)	CATLINS (n=48)	TAUTUKU (n=5)	STEWART ISLAND (n=47)
Brachyura	51.6	0.6	54.9	40.4	70.0
Amphipoda	36.1	80.0	1.6	23.2	0.4
Anomura	-	-	-	36.4	0.6
Polychaeta	9.6	1.9	10.6		4.7
Bivalvia	0.8	10.3	4.4		5.3
Teleostei	<0.1	-	9.9	-	3.4
Pericarida	<0.1	-	-	-	7.9
Eggs	-	-	7	-	-
Crustacea	0.5	1.6	3.4	-	0.7
Decapoda	0.3	0.3	5.2	-	-
Balanomorpha	-	0.7	-	-	4.6
Diptera	-	2.8	-	-	-
Rhodaphyta	-	-	2.6	-	-
Algae	0.5	-	0.1	-	0.5
Isopoda	<0.1	1.8	-	-	0.2
Tanaida	-	-	-	-	0.8
Nemertea	-	-	-	-	0.7
Pycnogonida	0.6	-	-	-	-
Archaegastropoda	<0.1	-	<0.1	-	<0.1
Euphausida	<0.1	-	-	-	0.1
Gastropoda	-	-	-	-	0.1
Coleoptera	-	-	-	-	<0.1

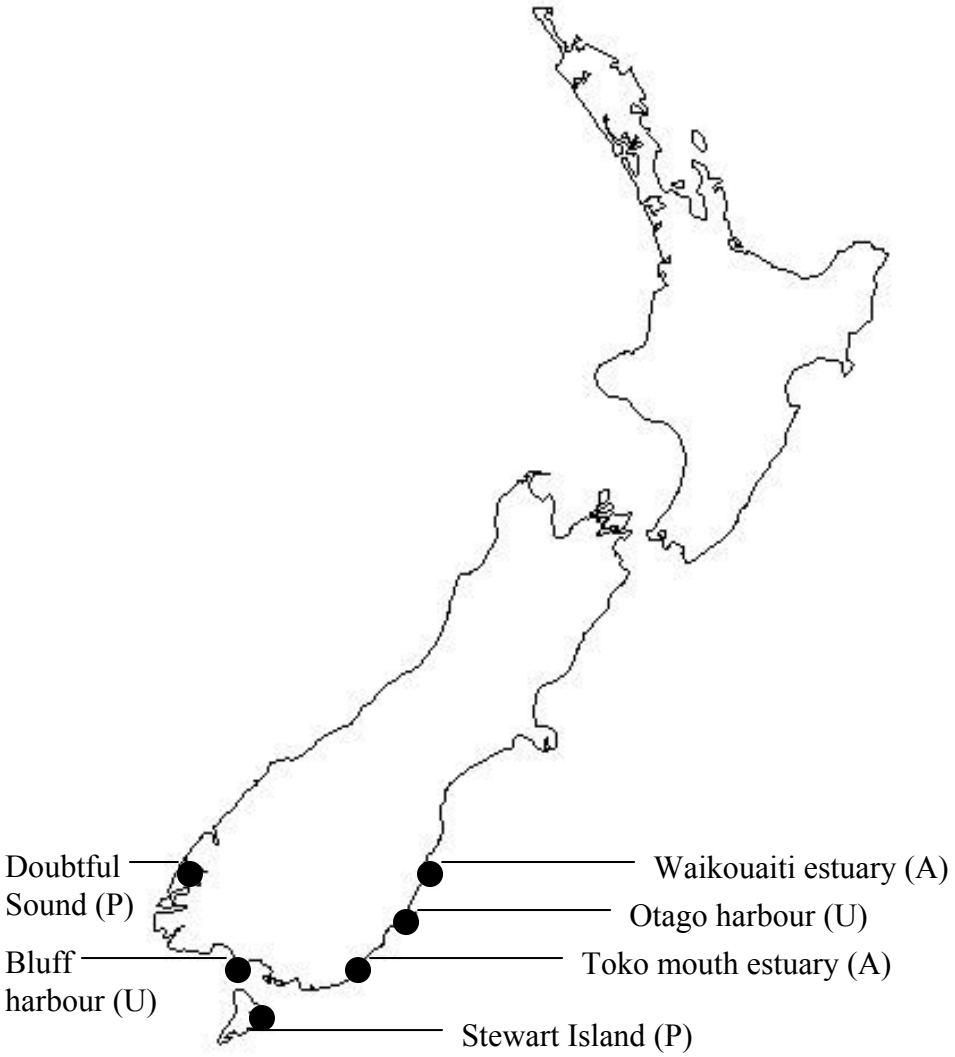


Figure 1: Map of New Zealand showing the six study areas on South Island and their dominant land use type: A, agricultural; U, urban watershed; P, pristine forested catchment.

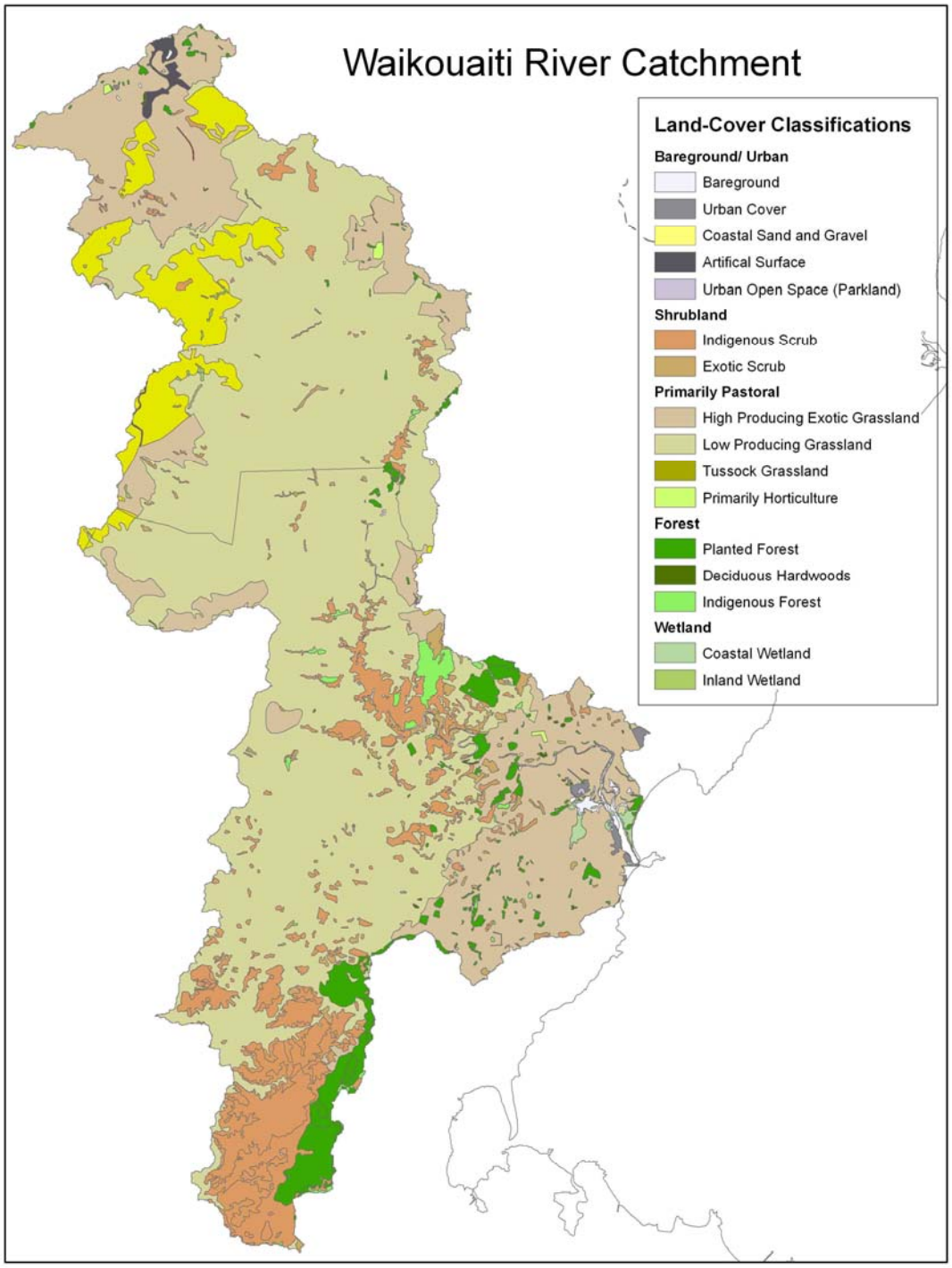


Figure 2: Land use in the Waikouaiti river catchment classified into 16 categories according to the LCDB2 categories of land use. The receiving water bodies are influenced predominantly by agricultural inputs as shown by the areal extent of pastoral land (shown in shades of beige).

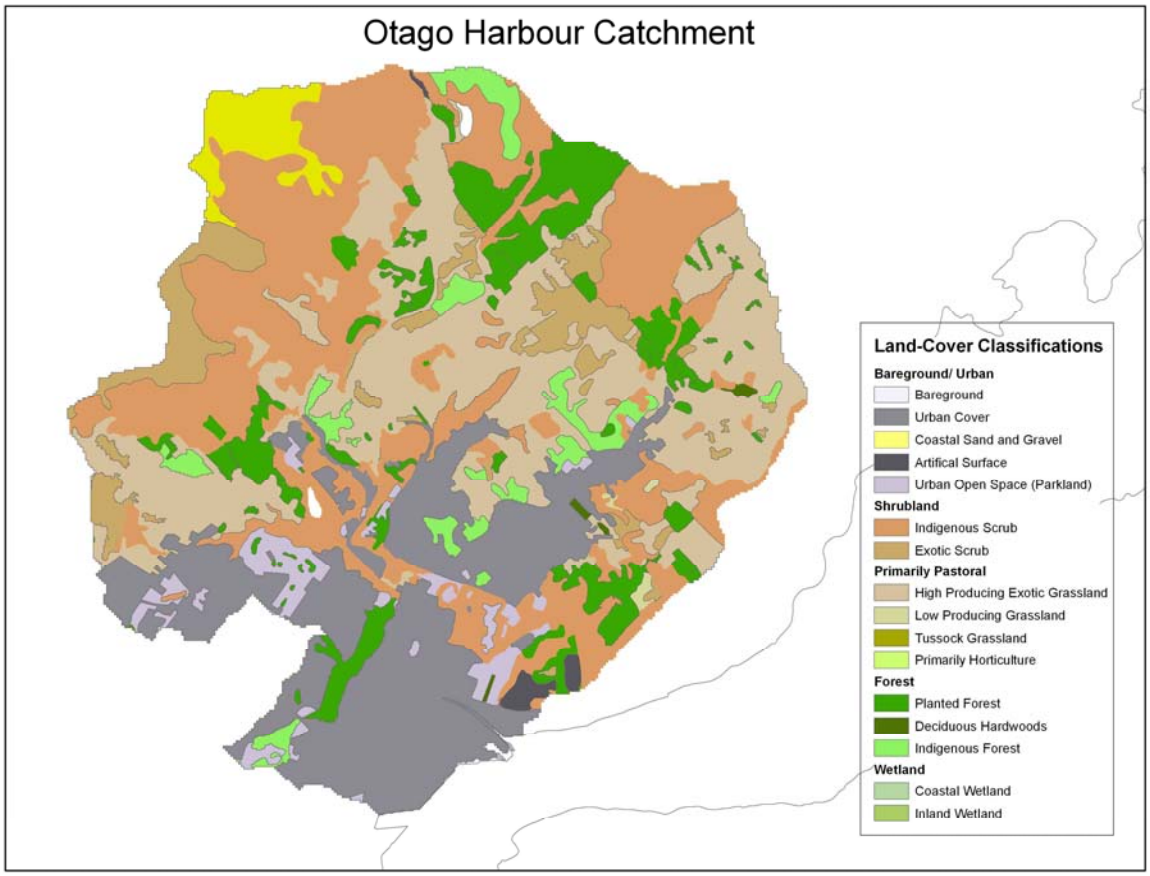


Figure 3: Land use in the Otago Harbour catchment classified into 16 categories according to the LCDB2 categories of land use. The inner harbour basin is influenced by run-off from multiple nutrient inputs associated with urban development. The catchment is characterised by impervious surfaces (roads; shown in grey) and a mosaic of shrubland (brown) and pastoral land (beige).

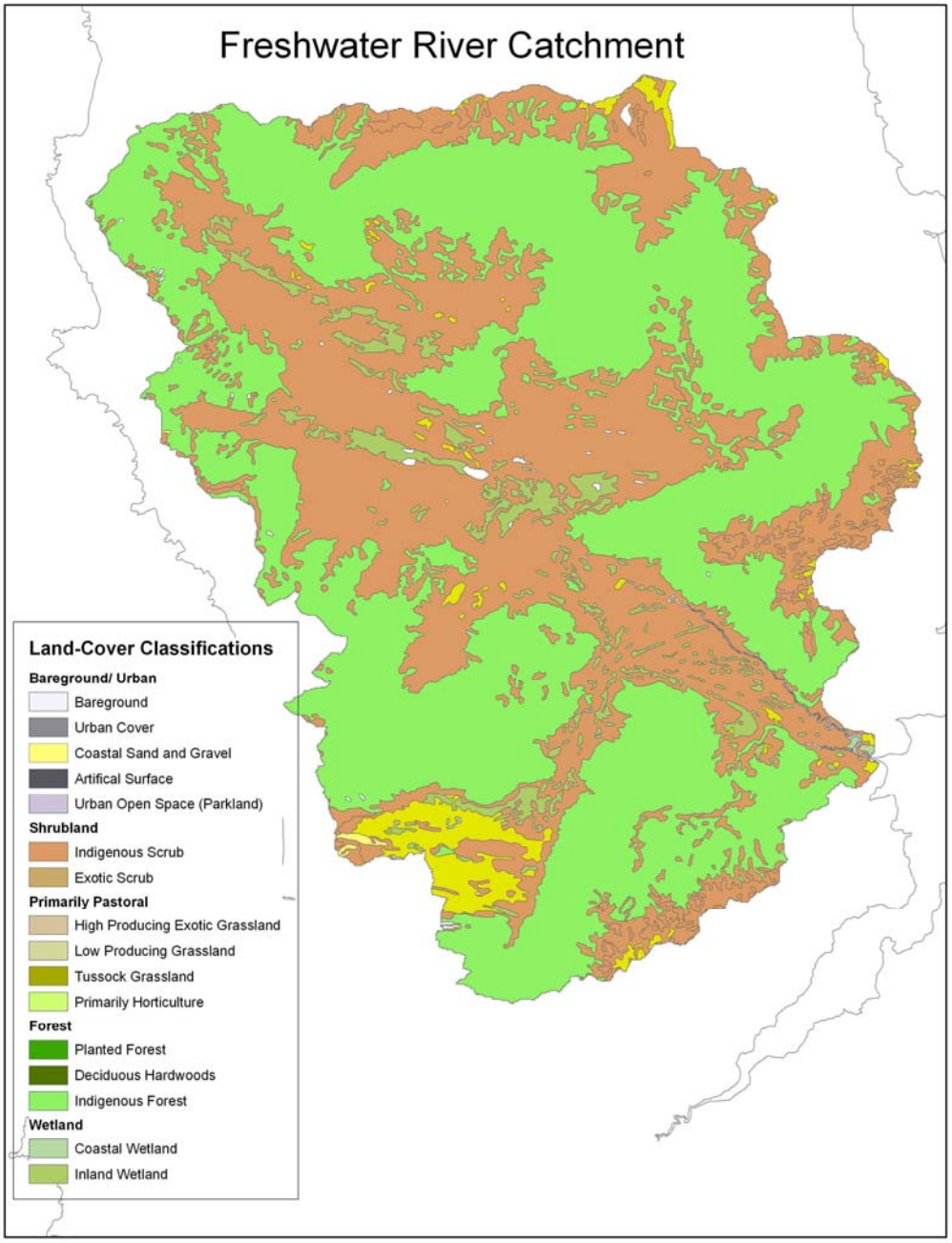
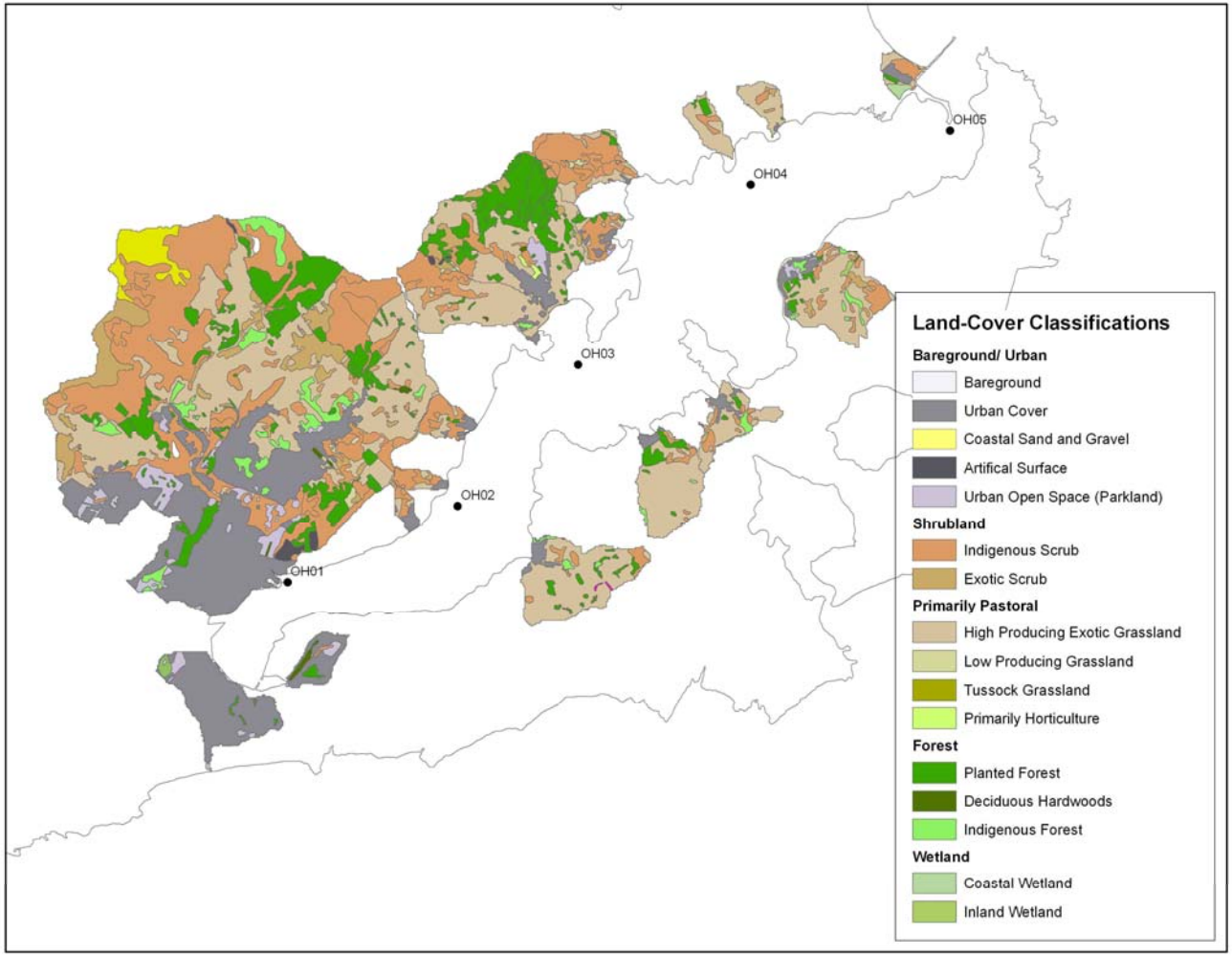


Figure 4: Land use in the Freshwater river catchment (Stewart Island) classified into 16 categories according to the LCDB2 categories of land use. The drainage basin is dominated by native forests and shrubs (shown in green and brown, respectively).

Figure 5a: Catchments with complex drainage patterns and multiple nitrogen source pools were also analysed using multiple subcatchments where nitrogen loads to individual sites were calculated from upstream subcatchments. In this example, the multiple catchments surrounding Otago Harbour were delineated in the aerial photograph before associated land cover classifications were quantified using GIS (Figure 5b).

Figure 5b: Catchments with complex drainage patterns and multiple nitrogen source pools were also analysed using multiple subcatchments where nitrogen loads to individual sites were calculated from upstream subcatchments. In this example, the land cover classifications of the multiple catchments surrounding Otago Harbour were quantified using 16 LCDB2 categories. The five sampling sites in Otago Harbour (OH01-OH05) where water samples and biota were collected are also illustrated in the GIS map.



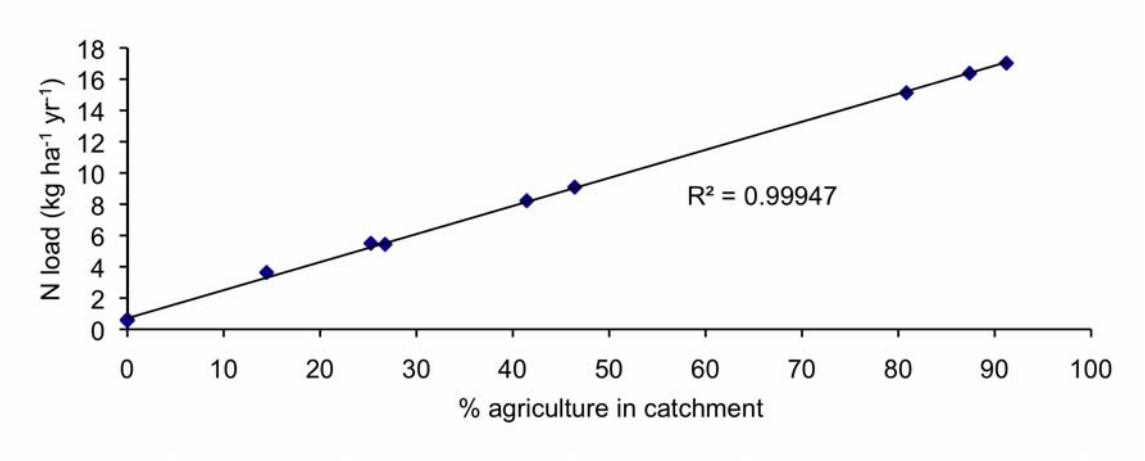
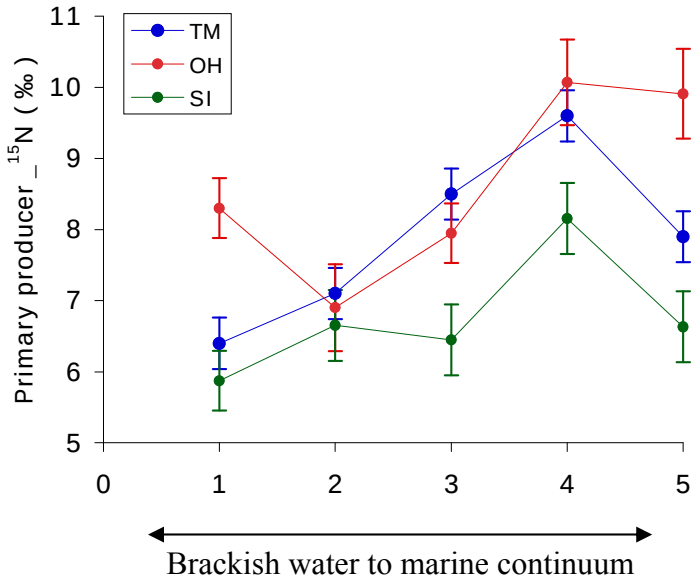


Figure 6: Relationship between nitrogen loading rates (kg ha^{-1} yr^{-1}), normalised to watershed area, and percent agriculture in catchment. The data are from subcatchments within the Otago Harbour, Stewart Island, and Waikouaiti coastal habitats (Table 2).

a



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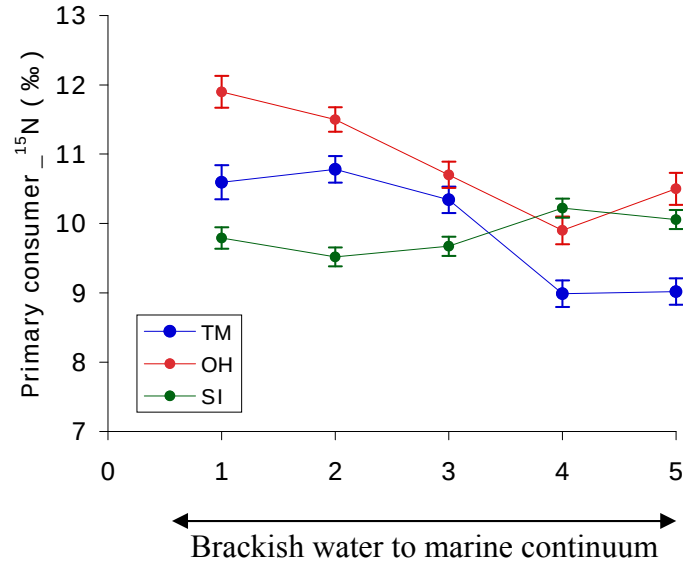


Figure 7: Gradients in $\delta^{15}N$ values across individual coastal ecosystems for (a) primary producers (*Ulva* spp.) and (b) primary consumers (*Austrovenus stutchburyi*). Individual data points represent site-specific mean $\delta^{15}N$ values ± S.E. (n=5) for spring, the growth period. The data are from an agriculture dominated estuary, Toko mouth (TM, in blue); urban coastal inlet, Otago Harbour (OH, in red); and pristine estuary, Stewart Island (SI, in green). Note the different scales on the y-axis. The x-axis refers to sites within each coastal ecosystem, from site 1, the innermost brackish water site, to site 5, the outermost fully marine site of each system.

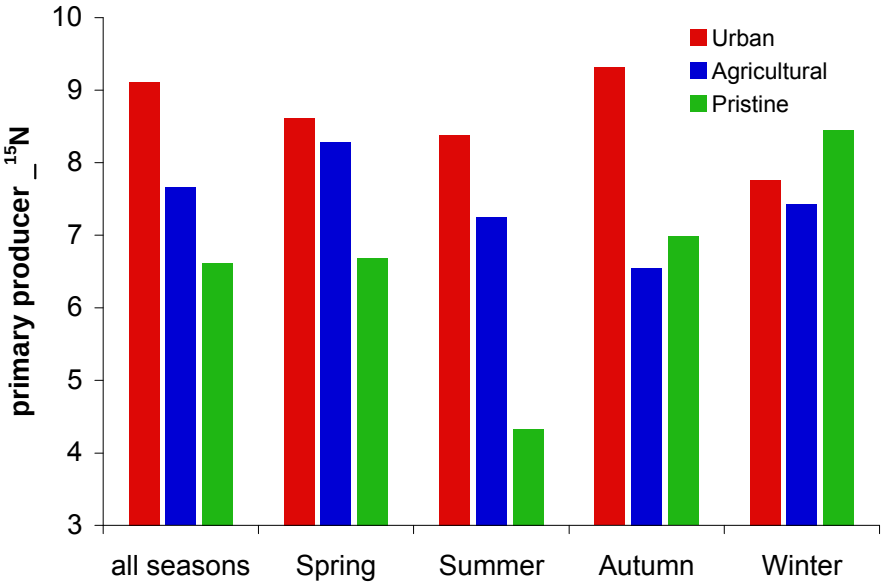


Figure 8: Seasonal shifts in primary producer mean $\delta^{15}N$ values for coastal habitats influenced by different surrounding land use practices (urban, agricultural, and pristine catchments). Data are mean $\delta^{15}N$ values for *Ulva* spp. collected across each study area and include isotope ratios for sites 01-05 (n=25 at least per season). All seasons refers to the average $\delta^{15}N_{Ulva}$ for all seasons combined (n=100 per location).

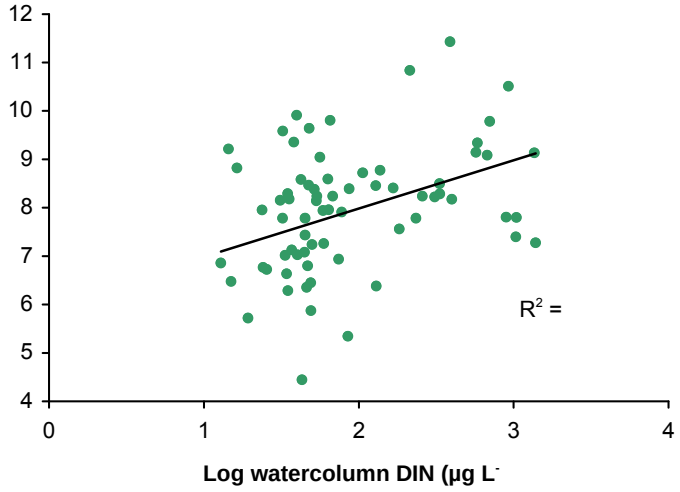
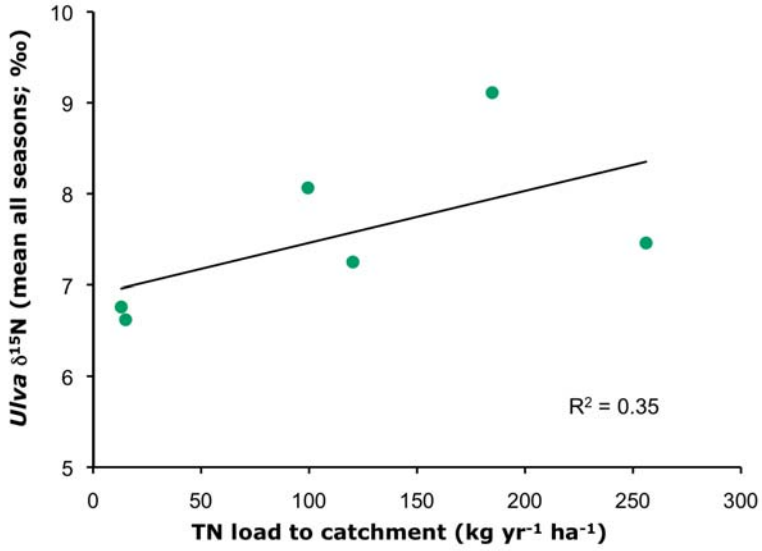


Figure 9: Relationship between macroalgal (*Ulva* spp.) $\delta^{15}N$ values (‰) and water column dissolved inorganic nitrogen (DIN) loads (µg L^{-1}) measured across all sites.

a



b

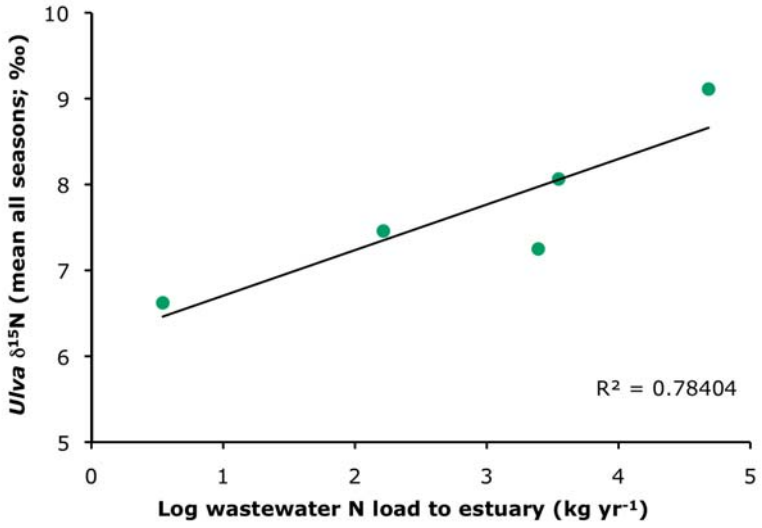


Figure 10: Relationship between macroalgal $\delta^{15}N$ values (‰) averaged for all seasons, and (a) modelled total annual nitrogen (TN) loads (kg yr^{-1} ha^{-1}) entering each study area, standardised per catchment area; and (b) modelled wastewater N loads to each estuary.

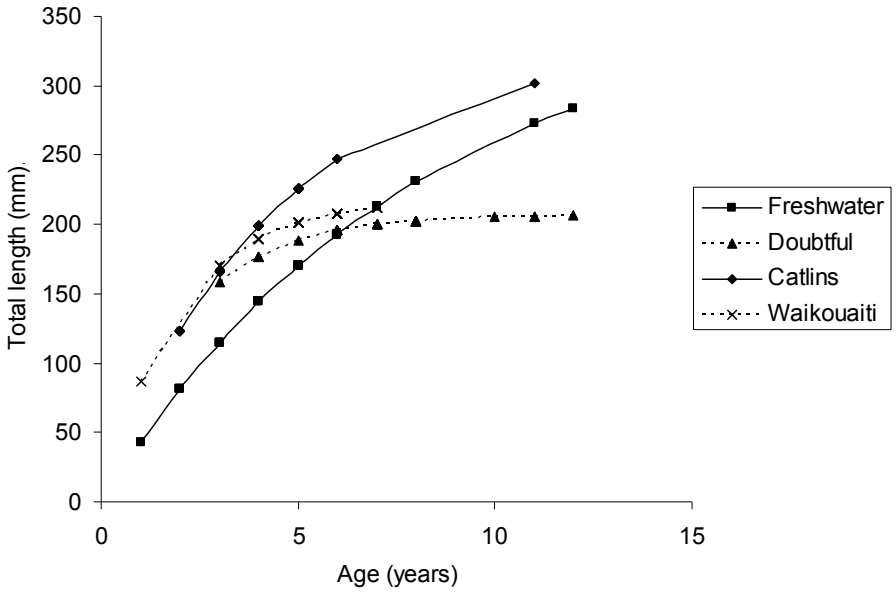


Figure 11: Von Bertalanffy growth curves for spotties (*Notolabrus celidotus*) from the Waikouaiti, Catlins and Freshwater river (Stewart Island) estuaries and Doubtful Sound based on otolith aging.

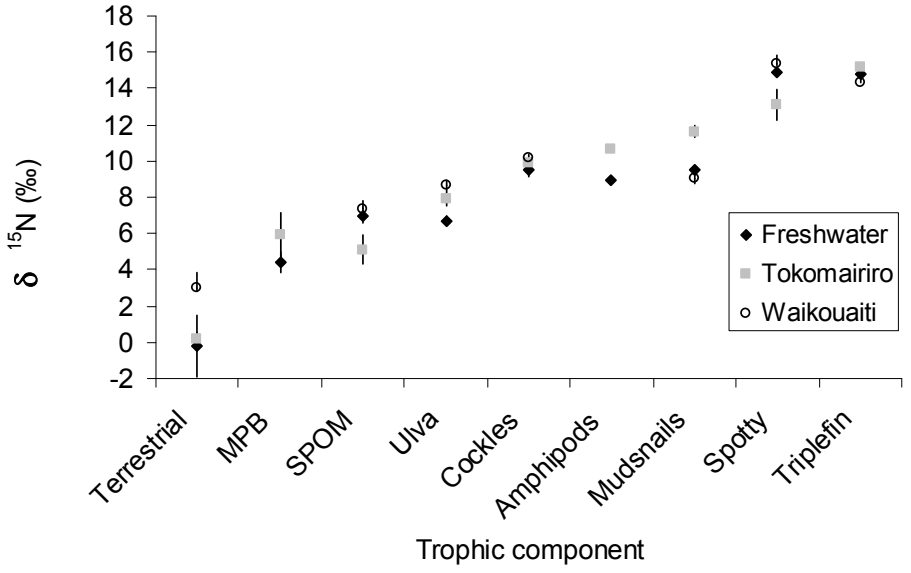


Figure 12: $\delta^{15}N$ values for key components of the Freshwater (Stewart Island), Waikouaiti, and Toko mouth estuarine food webs. Evidence exists for the enrichment of the benthic food web of the most nutrient-enriched estuary (Tokomairiro) relative to the moderately-enriched estuary (Waikouaiti) and the pristine area (Freshwater). Error bars indicate ± 1 S.E.

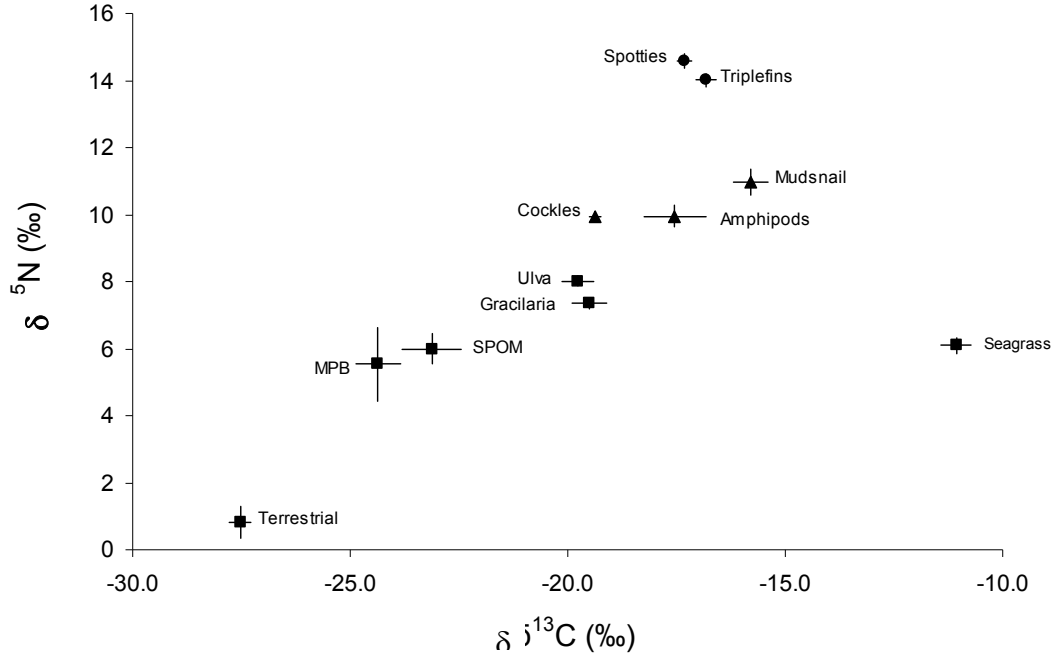


Figure 13: Generalised food web for southern New Zealand estuaries based on $\delta^{15}N$ and $\delta^{13}C$ signatures of key components and culminating in triplefins and spotties. Primary producers are represented as squares, primary consumers as triangles, and secondary consumers as circles. Error bars represent ± 1 S.E. n = 16-110 for the different food web components. MPB, microphytobenthos; SPOM, suspended particulate organic matter.

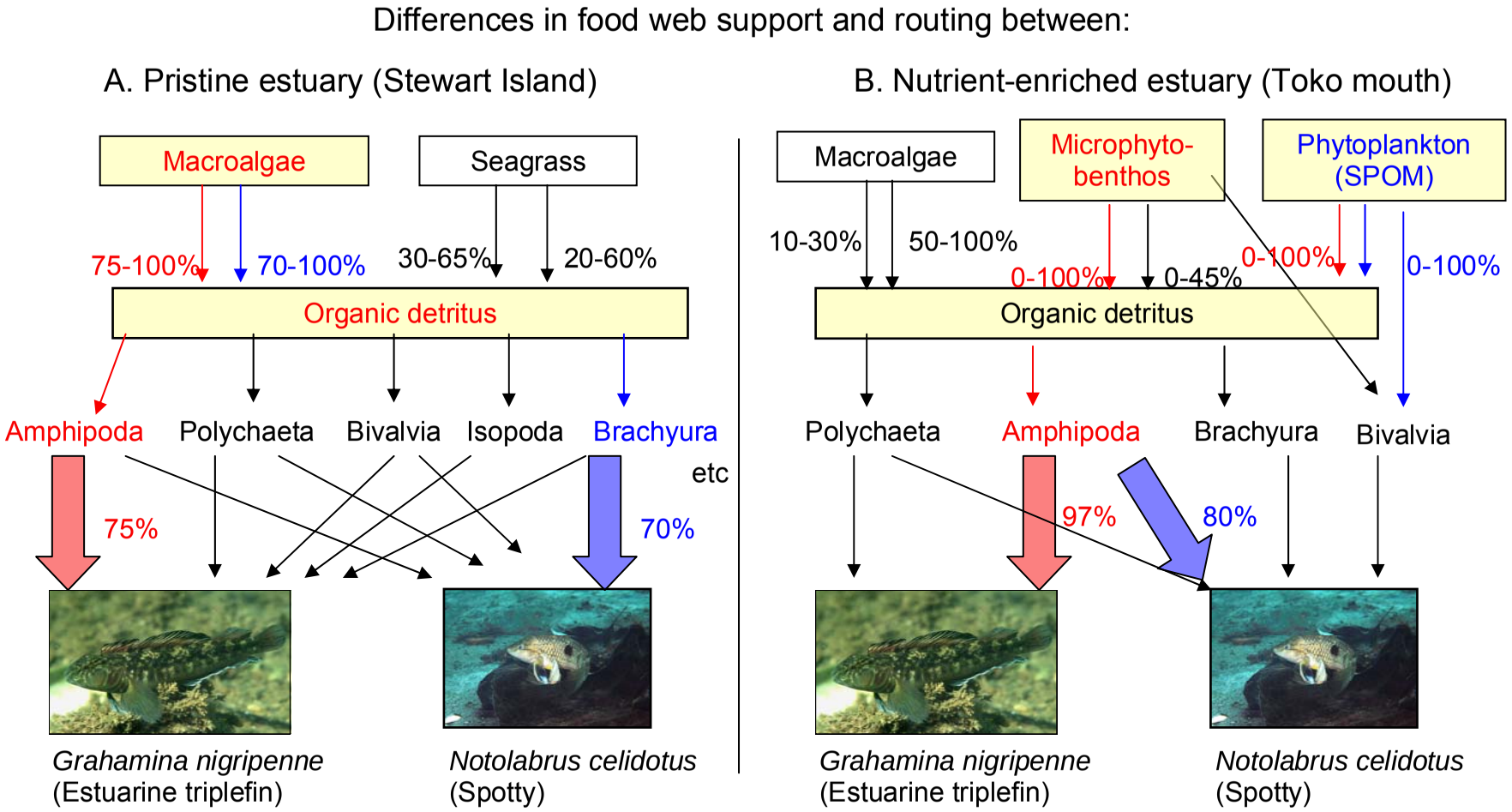


Figure 14: Schematic showing the differences in food web support (carbon sources) and routing in a pristine estuary (Stewart Island) and a nutrient-enriched estuary (Toko mouth) based on dual stable isotope ratios and gut content analyses. The dominant prey items of triplefins and spotties are derived from %IRI from gut content analyses and highlighted in red and blue. The numbers with respect to the primary producers refer to percent relative importance based on the outputs of the two-source stable isotopic mixing models and varies seasonally and for the different study areas.