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Ecological alterations associated with Submarine Groundwater Discharge (SGD), utilising Stable Isotope Analysis (SIA)

A Thesis Presented to the Academic Faculty in Partial Fulfillment
of the Requirements for the Degree

Doctor of Philosophy

2014

Laura Judith Foley

Trinity College Dublin

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Summary

Research was conducted in three systems over two ecoregions to examine the ecological impacts associated with SGD. The potential applications of stable isotope analysis (SIA) in the field of SGD ecological research were investigated and some of the possible causative physicochemical agents of ecological change associated SGD were considered. Two study systems involved karst-channeled SGD (Ohlos de Água, an exposed oceanic rock pool platform in southern Portugal; and Kinvara bay, a sheltered, semi-enclosed bay on the west coast of Ireland). The third system was the sheltered, semi-enclosed Ria Formosa lagoon in southern Portugal, where diffuse SGD issues from a sandy beach face. The research involved *in-situ* ecological surveys, macroalgal and macroinvertebrate sampling and characterisation, water chemistry analysis, organism N and C elemental analysis and SIA.

In all systems, SGD was associated with ecological alterations. The type of alteration differed, even when comparing two instances of karst-channeled SGD. The presence of SGD was associated with altered macroalgal composition and biomass (Ria Formosa lagoon (p = 0.003) and Kinvara bay (p = 0.008)), alterations to the macroinvertebrate assemblages (Ria Formosa lagoon (p = 0.002 for abundance, p = 0.06 for biomass) and Kinvara bay (p = 0.008)), and changes in sessile species composition (Kinvara bay (p = 0.008), and Olhos de Água rock pools ($p < 0.0001$)).

In all cases, SGD was nitrogen-enriched (Kinvara - up to 90 µM; Olhos de Água - up to 555 µM, and from other research in the Ria Formosa - up to 265 µM). In all cases SGD contained a freshwater component (from results documented herein for Kinvara bay (salinity 0) and Olhos de Água (salinity 4 recorded), and those documented elsewhere for the Ria Formosa (salinity 17 recorded)). In Kinvara bay, SGD had altered temperature (warmer in winter, cooler in summer) relative to the marine compartments (11.3 °C vs.10 °C, and 11.9 °C vs. 15 °C, resp.). It is suggested that the influence of SGD over ecology was conditioned primarily by the salinity regime imposed by SGD, enhanced in two systems (Ria Formosa lagoon and Kinvara bay) by nitrogen additions. Comparison of the two instances of karst-channeled SGD illustrated the relevance of this form of SGD in particular in coastal nitrogen loading, even in systems associated with catchments subject to relatively low levels of anthropogenic nitrogen pollution. This is due to the relatively conservative nature of nitrate transport in these systems.

SGD may act as a disturbance comprising salinity (a natural stressor), and, in cases, anthropogenic nitrogen loading (an anthropogenic stressor). In the Ria Formosa lagoon, SGD occurred as a disturbance of intermediate frequency and resulted in an increased primary producer species list at the SGD compared to the control sites. In Kinvara bay on the other hand, the frequency of disturbance caused by SGD was relatively low, leading to a reduced macroalgal species list and high biomass of those species present (greater than five times the macroalgal biomass relative to control sites). In the third system, SGD resulted in a very high frequency of disturbance, maintaining a reduced species list (3.25 ± 0.48 in rock pools vs. 3.75 ± 0.25 in control rock pools, (mean ± SE) $n = 4$, n.s.) as well as relatively low biomass of species at the SGD sites.

SIA of nitrate in water (Kinvara) and N and C of food web components (Ria Formosa and Kinvara) allowed the trophic transfer of SGD-borne N and C to be traced into and through food webs in the immediate vicinity of intertidal submarine groundwater discharge. This illustrated the existence of two discrete food webs (one in the vicinity of SGD, and one not in the immediate vicinity of SGD) with unique pathways of trophic transfer. $\delta^{15}N$ analysis was used to tentatively infer (a) some influence of a groundwater source, and a synthetic fertilizer N source contained in the groundwater in the Ria Formosa due to food web component ^{15}N depletion (by ~0.05 ‰), and (b) some contribution of N from a septic tank effluent/wastewater N source to the SGD food web in Kinvara bay due to algal and macroinvertebrate ^{15}N enrichment (~0.05 ‰ and ~1.5 ‰ respectively). Enrichment of the SGD food web in ^{13}C (by ~2 ‰) was used to infer increased rates of carbon turnover and primary producer respiration at SGD sites in the Ria Formosa lagoon. Also, depletion of ^{13}C in the SGD food web (by ~6 - 7 ‰) was used to indicate SGD as a source of C loading to Kinvara bay. SGD algae samples in Kinvara bay in winter were very depleted in ^{13}C (~- 36 ‰). This distinct signature may enable researchers to trace the impact of SGD into the wider ecosystem and investigate its effects on the functioning and provision of ecosystem goods and services within the wider ecosystem. In both systems, $\delta^{13}C$ was a more useful tracer than $\delta^{15}N$ due to greater divergences in carbon isotopic signature at the base of the food web (i.e. macroalgae here). Save one comparison, analysis of elemental data (C, N and C:N) did not provide any significant results, indicating the superior utility of SIA in such research.

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Acknowledgements

This work is dedicated to my father. Without him this Ph.D. would not have been possible. Dad always demonstrated an admirable work ethic, enthusiasm for learning and incredible resiliance. He instilled in me his curiosity and inquisitiveness. Above all, I owe my father my loving thanks for his uncompromising, unconditional support.

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List of abbreviations

AMBI	AZTI Marine Biological Index
BI	Biotic Index
C	Elemental Carbon
^{13}C	Carbon 13 isotope
$\delta^{13}C$	Deviation in carbon isotopic ratio ($N^{15}:N^{14}$) from the standard, Pee Dee Belimnite
CCM	Carbon Concentrating Mechanisms
D	Simpsons index (diversity index)
DIC	Dissolved Inorganic Carbon
$gCm^{-2}y^{-1}$	Grams of carbon per meter squared per year
H'	Shannon-Wiener index (diversity index)
H_2O	Water
HCO_3^-	Bicarbonate
Km^2	Kilometer
$m^3 s^{-1}$	Cubic meters per second
M-AMBI	Multivariate-AZTI Marine Biological Index
mgm^{-2}	Milligrams per meter squared
mm	Millimeter
MPB	Microphytobenthos
N	Elemental Nitrogen
^{15}N	Nitrogen 15 isotope
$\delta^{15}N$	Deviation in nitrogen isotopic ratio ($N^{15}:N^{14}$) from the standard, atmospheric nitrogen
NH_4^+	Ammonium
$NO2^-$	Nitrite
NO_3^-	Nitrate
OGM	Opportunistic Green Macroalgae
O_2	Oxygen
pCO_2	Partial pressure of carbon dioxide
pH	Power of the hydrogen ion
psu	Practical Salinity units
S	Species richness (diversity index)
SGD	Submarine Groundwater Discharge
SIA	Stable Isotope Analysis
µM	Micromole
WFD	Water Framework Directive

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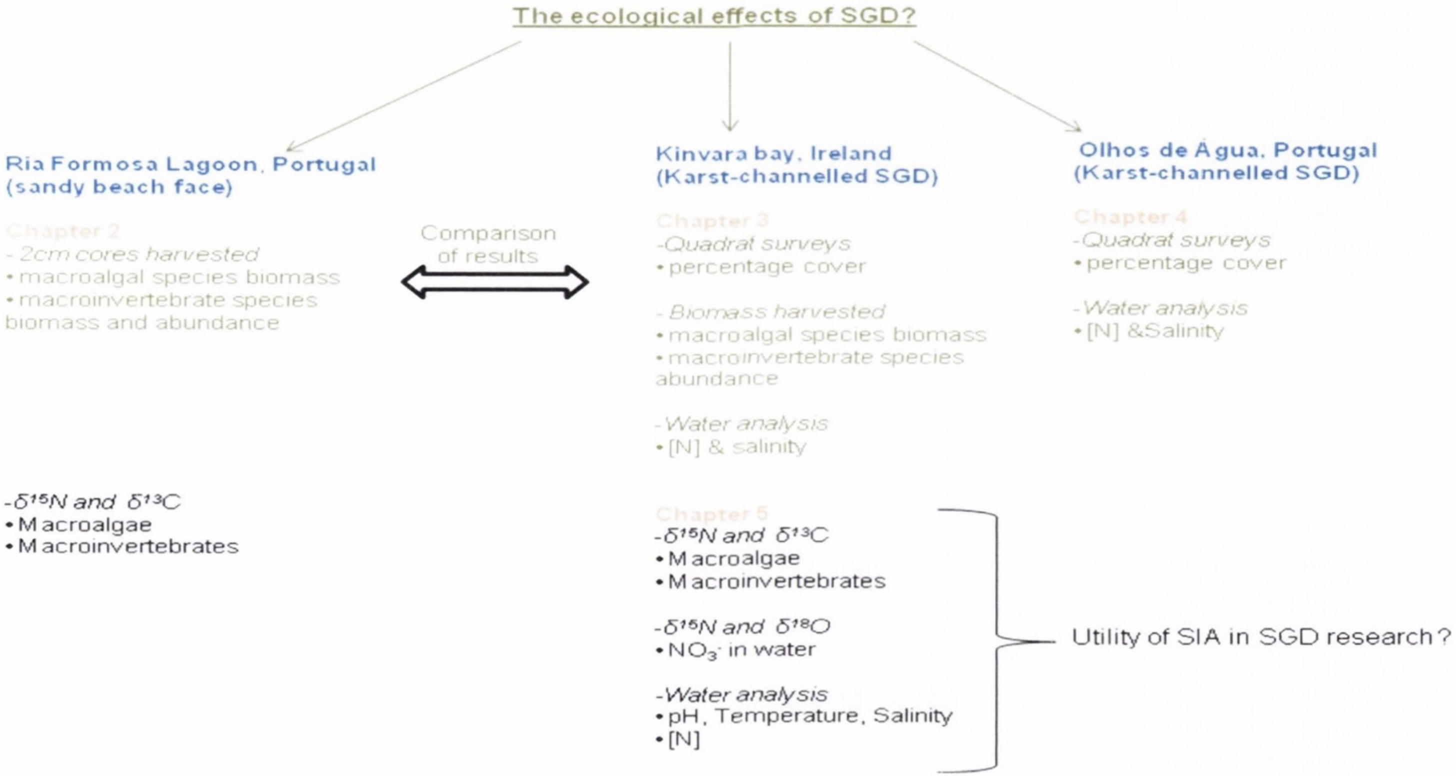
Research Overview



The ecological effects of SGD?

Ria Formosa Lagoon, Portugal (sandy beach face)

Chapter 2

- 2cm cores harvested
- macroalgal species biomass
- macroinvertebrate species biomass and abundance

-$\delta^{15}N$ and $\delta^{13}C$
- Macroalgae
- Macroinvertebrates

Comparison of results

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-Quadrat surveys
- percentage cover

- Biomass harvested
- macroalgal species biomass
- macroinvertebrate species abundance

-Water analysis
- [N] & salinity

Chapter 5

-$\delta^{15}N$ and $\delta^{13}C$
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-$\delta^{15}N$ and $\delta^{18}O$
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-Water analysis
- pH, Temperature, Salinity
- [N]

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Chapter 4

-Quadrat surveys
- percentage cover

-Water analysis
- [N] &Salinity

Chapter 1. General introduction

1.1. Coastal ecosystems: functioning, pollution, goods and services

Many ecosystem services, i.e. the processes and conditions of natural ecosystems that support human activity and sustain human life (Millennium Ecosystem Assessment 2005, p.155) derive from marine and in particular, coastal ecosystems (Costanza et al. 1997). The term 'Ecosystem Functioning' refers to the flow of energy and materials through the biotic and abiotic components of the ecosystem (Díaz and Cabido 2001). Sustained, balanced ecosystem functioning underpins the continued provision of ecosystem goods and services. 'Biodiversity', which refers to the variety of life, including ecosystem diversity, species diversity and genetic diversity within a species (CBD 2012), is rapidly being altered globally, often negatively (Cardinale et al. 2012; Loreau et al. 2001). Biodiversity enhances ecosystem reliability (Naeem and Li 1997) and stability (Basset et al. 2013) and its loss can significantly affect ecosystem functioning (Cardinale et al. 2012) and compromise the continued provision of ecosystem goods and services which underpin the quality of human life (Worm et al. 2006; Costanza et al. 1997).

One of the main causes of altered biodiversity in marine systems is eutrophication, a worldwide phenomenon (Howarth et al. 2000) and a major threat to the balanced functioning of aquatic systems (Diaz and Rosenberg 2008, p.2). Though phosphorus concentrations are of importance, nitrogen (N) is normally the limiting nutrient in marine systems (Howarth and Marino 2006a; Howarth et al. 2002; Nixon 1995; Paerl 1997). Excessive loading of nitrogen in coastal, estuarine and open marine systems can lead to eutrophication. There are many formal definitions of 'eutrophication', but possibly the most widely used is the one coined by Nixon (1995). This definition considers eutrophication as a process; '*eutrophication is an increase in the rate of supply of organic matter to an ecosystem* (Nixon 1995)'. This increase in supply of organic matter to coastal systems may have various causes/sources (primary production of higher plants and benthic microalgae as well as inputs of organic matter from adjacent waters or from land, via rivers or point source), but the common factor is nutrient enrichment (Andersen et al. 2006; Heisler et al. 2008; Howarth et al. 2000; Nixon 1995). In light of this acknowledged common 'nutrient' factor, coastal eutrophication is sometimes considered and defined in terms nutrient enrichment and/or the subsequent effects of the increased rate of supply of organic matter in the ecosystem. For example, the European Commission (EC) Nitrates Directive (91/676/EEC) defines coastal eutrophication as '*the enrichment of water with nitrogen compounds causing accelerated growth of algae and higher forms of plant life to produce an undesirable disturbance to the balance of organisms present in the water and to the quality of the water concerned*' (Anonymous 1991). A comprehensive definition thus includes all three components - increased organic matter supply (either autochthonous or allochthonous) associated with nutrient loading, and the subsequent ecosystem effects.

Eutrophication can be a primary driver of decreased water quality and habitat degradation, potentially over large geographical areas and extended periods of time. When it persists it usually culminates in invertebrate and fish mortality (Diaz and Rosenberg 2008) and leads to changes to community composition (Cohen and Fong 2006). It adversely affects numerous ecosystem goods and services including fisheries, the provision of nursery habitats (e.g. oyster reefs, seagrass beds, wetlands), and filtering and detoxification services provided by suspension feeders, submerged vegetation and wetlands (Worm et al. 2006).

Eutrophication involving blooms of algae that produce harmful toxins (i.e. harmful algal blooms (HABs)) can result in mass mortalities of fish, seabirds and other marine life. These toxins can enter the human food chain (usually through the consumption of shellfish), causing sickness, particularly in vulnerable coastal communities of less developed countries. The decay of primary producers following eutrophication and HABs can culminate in areas of hypoxia unable to support life, known as 'dead zones'. The observed incidences of these 'dead zones' have been on the increase since oceanographers first began noting them in the 1970s. When the United Nations (UN) Environment Program published its first Global Environment Outlook Year Book in 2003, 146 dead zones were reported (UN 2003). By 2008, this had risen to 405 worldwide (Diaz and Rosenberg 2008).

Potential anthropogenic nitrogen sources are many (manure, slurry, sewage, synthetic fertilizers, waste disposal sites, industrial waste etc.). The method of delivery of the sources to the marine system can be point or diffuse (localised point of release versus non-localised point of release). Due to their proximity to anthropogenic nitrogen sources, coastal marine systems are particularly vulnerable to eutrophication driven by anthropogenic nitrogen enrichment. The process of eutrophication is a major concern in Irish tidal waters (EPA 2010, p.115). Over half of the 89 coastal and estuarine water bodies assessed in the most recent Irish Water Quality Status assessment and report for the period 2007-2009 were by impacted eutrophication (O'Boyle et al. 2010). In terms of surface area, 102.1 km^2 or 5.3 % of the total area assessed (just under 2,000 km^2) was classed as either eutrophic or potentially eutrophic (O'Boyle et al. 2010). This is not an issue particular to Ireland but one that is mirrored throughout European coastal and estuarine systems, including the Ria Formosa lagoon in southern Portugal (Newton et al. 2003).

It is necessary to evaluate all potential pathways of anthropogenic contaminants, particularly nitrogen, to the marine environment, and further, to characterize the form and magnitude of ecological alterations associated with each loading source. A global lack of estuarine response to land remediation efforts (Loveless and Oldham 2010) underpins this need to completely understand the links and relationship between loads and the discharge environment. Furthermore, demands of the EC require that the 'polluter should pay' (97/11/EC) for mitigation and remediation of pollution events. Compliance with this requires the identification of the geographical origin and source of pollution, and information regarding the pollution pathway.

1.2. Submarine groundwater discharge (SGD)

Submarine ground water discharge (SGD) is defined as any water which moves from the seabed into the coastal ocean, regardless of fluid composition or driving force (Burnett et al. 2003), over a scale length of meters to kilometers (Moore 2010). This discharge can comprise any one of three constituent water parcels, recirculated seawater, fresh groundwater or a brackish mixture of both (Burnett et al. 2003) (Figure 1-1). The occurrence of SGD is globally, and mostly intertidal zone (Figure 1-2).

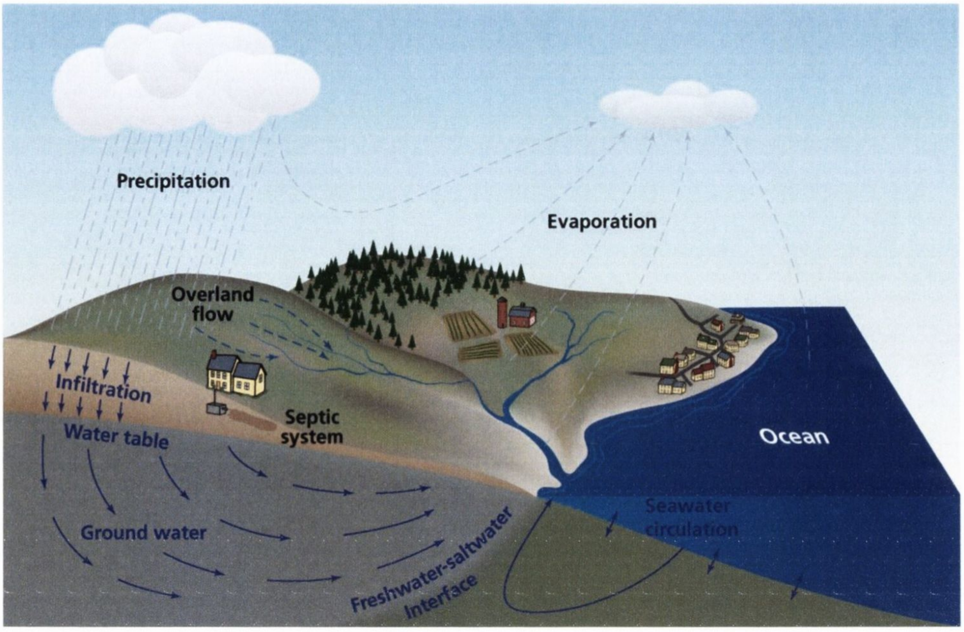


Figure 1-1 Schematic mode of occurrence of submarine groundwater discharge, illustrating fresh intertidal SGD and recirculated seawater SGD, adapted from Cook (2005).

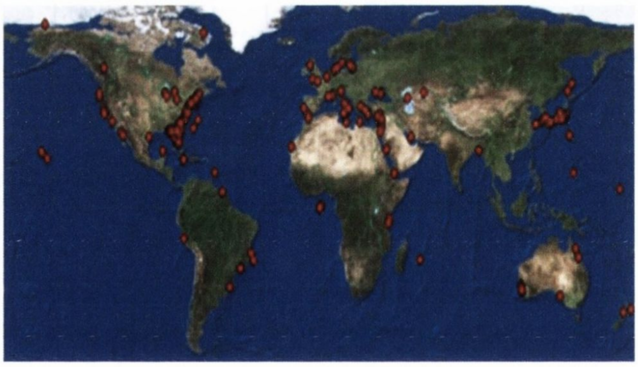


Figure 1-2 Documented occurrence of submarine groundwater discharge up to 2007. Adapted from the International Atomic Energy Agency (IAEA 2007).

The submarine discharge of groundwater is a natural phenomenon, however, it becomes of human concern when it is fuelled by fresh groundwater which is subject to anthropogenic pressure, e.g. extraction or pollution. The fresh groundwater compartment of SGD can deliver large fluxes of freshwater to marine environments, and is estimated to be responsible for as much as 6 - 10% of total global oceanic freshwater inputs, and regionally potentially accounting for 100% of freshwater inputs to the marine environment (see review by Taniguchi 2002). This freshwater portion of SGD can provide an important pathway for terrestrial nutrients and other dissolved species to the marine environment (Slomp and Van Cappellen 2004), representing a source of biogeochemically important new nutrients to the ocean, particularly at the intertidal zone (Burnett et al. 2003). The concentrations of nutrients in groundwater can be several orders of magnitude greater than that of surface water bodies and elevated SGD nutrient (particularly nitrogen) concentrations are frequently recorded (Hays and Ullman 2007; Kroeger et al. 2007; Dürr et al. 2008; de Sieyes et al. 2008; Carvalho et al. 2013; Ibánhez et al. 2013). Quantitative studies on groundwater nutrient inputs to the coastal zone and comparisons at a range of scales indicate that chemical loads to coastal waters from SGD commonly rival those from riverine transport (Taniguchi 2002; Slomp and Van Cappellen 2004; Kroeger et al. 2007).

All three forms of SGD have the potential to alter the chemical balance of the receiving marine environment. Recirculated seawater can release previously bound autochthonous ions including nutrients (nitrate, ammonia, phosphate) and through this can significantly alter the chemical composition of the discharging water (Moore 1999; Slomp and Van Cappellen 2004). The freshwater compartment of SGD serves as a potential pathway for new nutrients and dissolved species to the marine environment (Slomp and Van Cappellen 2004). Though both compartments (seawater and freshwater) can deliver nutrients, the freshwater compartment is the one of concern as it may potentially deliver large and ecologically significant fluxes of allochthonous nutrients, especially N, to marine environment and littoral systems in particular.

1.3. Submarine groundwater discharge, nutrient enrichment and ecosystem responses

Determining the ecological effects of SGD nutrient loading in receiving coastal ecosystems is complicated by (1) issues around determining which nutrients are limiting to primary production within the receiving system and (2) differences in the way that coastal ecosystems respond to nutrient enrichment.

1.3.1. Nutrient limitation

The C:Si:N:P ratio found in phytoplankton throughout the oceans, and that which is required for phytoplankton growth, is 106:15:16:1, known as the Redfield ratio (Redfield 1958). Release from nutrient limitation by increased molar concentration(s) of the limiting nutrient(s), i.e. increases in those nutrients which are present in lower molar concentrations than the C:Si:N:P 106:5:16:1 requirement, may result in stimulation of primary production and possibly the eventual onset of eutrophication (Howarth and Marino 2006). Carbon is generally considered to be available in limitless supply in estuarine systems because the concentration of total dissolved inorganic carbon (DIC) is usually 1 to 2 orders of magnitude greater than that of inorganic nitrogen or phosphorus (Fogel et al. 1992). Thus it is the molar ratio of silicon, nitrogen and phosphorus present in a system which largely determines the form of the primary producer community. For example, diatoms require silicon for construction of their siliceous outer shell during growth. Increases in nitrogen and/or phosphorus without concomitant increases in silicon can cause a shift from diatom production toward the growth of dinoflagellate and flagellate taxa as these do not require silicon for growth (Radach et al. 1990).

Enrichment of coastal waters with P, and particularly N, generally alters the macroalgal community composition by progressive selection for fast-growing algae (phytoplankton, microphytobenthos and macroalgae) (Borum 1996; Cohen and Fong 2006). Nutrient enrichment promotes a shift in the community composition of primary producers from perennial macroalgae and seagrass dominance toward dominance by phytoplankton and eventually ephemeral macroalgae (Borum 1996). This change in composition reflects a change from nutrient limitation to light limitation (Duarte 1995). Increased phytoplankton and ephemeral algae biomass cause reduce light availability imposing light-limitation which can limit the growth of benthic macrophytes, particularly vascular plants. Nutrient enrichment has resulted in massive accumulations of fast growing macroalgae (Valiela et al. 1997), and a direct link between nutrient enrichment and reduced vascular plant abundance has been found in some temperate estuaries (Christensen et al. 1998; Dunton 1996). Nutrient enrichment (N, P and C) also tends to favor phytoplankton of larger cell size as increased surface area allows for greater nutrient uptake and diffusion rates (Finkel et al. 2009; Tortell et al. 2008). Studies have found that elevated dissolved CO_2 favors large-celled colonial diatoms as opposed to small-celled diatoms (Tortell et al. 2008). Iron limitation in combination with light limitation, on the other hand, can favor the growth of smaller-celled phytoplankton (Sunda and Huntsman 1997). Thus, the primary producer assemblage in eutrophic coastal environments is generally dominated by large-sized phytoplankton (Kiorboe 1993) which eventually give way to fast growing opportunistic emphemeral macroalgae (Borum 1996; Cohen and Fong 2006).

In freshwater systems, phosphorus is normally the limiting nutrient, while nitrogen normally limits autochthonous primary production in the marine environment (Howarth and Marino 2006a; Howarth et al. 2002; Nixon 1995; Paerl 1997). Surface estuaries in temperate latitudes have frequently been found to experience both P and N limitation, with P limiting spring growth and N limiting summer growth (Conley 1999; Cloern 2001; Paerl 2009). This complicates the issue of determining the limiting nutrient in coastal systems in receipt of SGD, as SGD can range in salinity from 0 to ~36 psu depending on the relative proportions of freshwater and recirculated sea water which it comprises. Before discussing the various responses of ecosystems to nutrient enrichment and the causes of observed differences in these responses, I will first discuss the nutrient dynamics associated with coastally and intertidally discharging SGD.

1.3.2. Submarine groundwater discharge nutrient dynamics

Submarine groundwater discharge can alter the absolute concentration of N, P and Si, and thus the relative concentration of N, P and Si in receiving marine environments. As a result of this, the molar concentration of N in SGD frequently exceeds the Redfield Si:N:P ratio (15:16:1) (Craig et al. 2010). The concentrations of nutrients found in SGD are determined firstly by the nutrient loading to the aquifer from terrestrial sources (natural or anthropogenic) and secondly by the subterranean transit path and aquifer characteristics. Due to the subterranean nature of the SGD flow path, the concentrations of nutrients delivered by SGD may differ from that delivered by a surface estuary of similar salinity and the same initial inland N and P concentrations. This is due to (1) the presence of nutrient assimilating primary producers in surface estuaries and their absence in subterranean estuaries and (2) the influence of the aquifer on nutrient attenuation/removal for SGD-borne nutrients. Primary producers remove nutrients, particularly nitrogen and phosphate from surface estuary water as the water flows towards the coast (Paerl 2009). This greatly reduces the concentrations of nutrients delivered to the coast by surface estuaries (Paerl 2009).

This mechanism of nutrient removal is absent in subterranean estuaries, leading to elevated nutrient concentrations in subterranean relative to surface estuaries.

Nitrogen and phosphorus may be removed from groundwater via mechanisms not associated with primary producer assimilation. Microbially mediated groundwater nitrogen removal can occur in anoxic aquifers, mostly via denitrification (Rocha et al. 2009; Slomp and Van Cappellen 2004), and possibly also via dissimilatory nitrate reduction to ammonia (DNRA) and/or annamox. Well oxygenated aquifers however prohibit such anoxia-dependent removal and generally favor relatively conservative nitrate transport (Slomp and Van Cappellen 2004). As SGD flows toward the coastline, dissolved phosphate may be removed via sorption to iron oxides or co-precipitation with dissolved aluminium, calcium or iron to mineral phases (Slomp and Van Cappellen 2004). This phosphate removal occurs in both oxic and anoxic aquifers, though less efficiently in the latter (Slomp and Van Cappellen 2004).

Despite the potential nitrogen removal capacity in anoxic aquifers, SGD is normally associated with elevated nitrogen concentrations (e.g. Carvalho et al. 2013; Leote et al. 2008; Mutchler et al. 2007; de Sieyes et al. 2008). The contrasting behaviour of nitrate and phosphate in oxic groundwater systems typically results in a strong increase in the N/P ratio along the groundwater flow path in such systems (Slomp and Van Cappellen 2004). SGD fed by oxygen depleted aquifers usually also has elevated nitrogen concentrations, however the elevation in N:P may not be as extreme as for well oxygenated aquifers.

Thus, the N/P ratio of SGD typically differs from that of surface estuaries of comparable salinities due to differences in primary producer assimilation and nutrient attenuation and removal during transit to the coast, particularly when associated with oxic groundwater systems, e.g. karst aquifers (Valiela et al. 1990). The Irish Environmental Protection Agency (EPA) recognises that where a significant proportion of surface water flow is derived from groundwater, increased nitrate concentrations in groundwater may contribute to eutrophication in surface waters, particularly in transitional and coastal waters (EPA 2010, p.30). SGD-associated nitrogen loading alone cannot however be used to indicate the resulting ecosystem responses as these are also modulated by other factors and processes (Cloern 2001).

1.3.3. Responses of coastal systems to nutrient enrichment

The potential ecosystem responses to coastal nutrient enrichment are complex and can be classified as 'direct' and 'indirect' responses (Cloern 2001). Direct responses are those concerning altered nutrient ratio (Si:N and N:P), altered structure and composition of the primary producer community and altered sedimentation of organic matter (Cloern 2001). The number of potential indirect responses is greater than the number of direct responses, with the direct responses determining the form of the indirect responses. Indirect responses include, for example, changes in water transparency, habitat quality and diversity, and community structure and composition of macroinvertebrates and higher organisms (Cloern 2001). It is difficult to predict how an estuarine or coastal system will respond to nutrient enrichment as different estuarine systems show different responses to nutrient enrichments/release from nutrient limitation. For example, the response of primary producer (and particularly phytoplankton) biomass to nutrient enrichment can differ. In some coastal areas, nutrient enrichment stimulates accumulation of phytoplankton biomass whereas in other systems, nutrient additions may cause changes in the macroalgal and macrophytobenthos biomass (Cloern 2001). Time series data has documented a link between nutrients and phytoplankton biomass in some systems (Chesapeake Bay, USA (Harding and Perry 1997)), however data from other sites has shown no correlation between phytoplankton biomass and nutrient additions (e.g. Ythan Estuary, Scotland (Balls et al. 1995)). Thus there may be inter-system differences in the effect of nutrient increase in the system and the system response in terms of primary producer biomass (Borum 1996).

Inter-system differences in response to coastal nutrient enrichment may be observed in the changes which occur in the plant (macroalgal and seagrass) community across systems. These different responses to nutrient enrichment across coastal systems are due to attributes of each system which work synergistically to modulate the response to nutrient loading (Cloern 2001). These attributes result in large differences in the rates and patterns of nutrient assimilation and cycling among coastal ecosystems (Cloern 1999). The characteristics will constrain (e.g. the Baltic Sea and Black Sea) or amplify (e.g. Ythan Estuary, Scotland) the responses (both direct and indirect) of coastal ecosystems to nutrient enrichment (Cloern 2001). Due to the effects of these attributes, some estuaries exhibit classic symptoms of acute eutrophication, including enhanced production of algal biomass while other estuaries maintain low algal biomass and primary production despite nutrient enrichment (Cloern 1999). This ability to modulate the response can be referred to as the 'nutrient filter' of the system, where the strength of the filter is system specific (Cloern 2001). This filter is underpinned by the inherent physical and biological properties of a system, which, combined, determine the sensitivity of an ecosystem to nutrient enrichment (Cloern 2001). These properties include but are not limited to; tidal energy of the system; horizontal transport efficiency in the system; optical properties which control light exposure of submerged plants; and biological regulators (Cloern 2001). These attributes act synergistically as a filter which modulates the responses which manifest following nutrient enrichment (Cloern 2001).

Tidal energy strongly affects the onset and progression of eutrophication (Cloern 2001). Primary producer responses to nutrient loading are generally greatest in areas with small tidal energy (Cloern 2001) with microtidal estuaries often more sensitive to eutrophication than macrotidal estuaries, following nutrient enrichment (Monbet 1992). The opposing buoyancy forces due to salinity stratification associated with salinity gradients in coastal ecosystems modulate phytoplankton responses to nutrient enrichment (Lucas et al. 1998). Horizontal transport mechanisms determine the residence time of water, nutrients and plankton within the coastal environment. The amount of horizontal transport modulates the effect of nutrient enrichment as primary production is controlled by the balance between nutrient loading and removal via transport processes (Cloern 2001). Physical attributes of the system such as wide, basin geography and level of restriction of water exchange, if any, control horizontal transport. Coastal systems with slow transport rates and long residence times tend to retain exogenous nutrients and therefore are less efficient filters than those systems with shorter residence times (Cloern 2001). The physical attributes of the system which determine the light exposure of submerged plants is another filtering feature as algae biomass production requires both nutrients and light (Lucas et al. 1998). In some estuaries, primary production shows a stronger correlation with measures of light than with nutrients, annually (Cloern 1999). Also, there can in some systems be a significant biological component of the filter, provided by benthic filter feeders (Cloern 2001). Phytoplankton removal by benthic filter feeders can be sufficient to counteract the rate of phytoplankton primary production, that is, there can be a strong grazer or top down control of the responses to enrichment (Meeuwig 1999).

1.3.3.1. Response to SGD nutrient enrichment

Due to the elevated N relative to P concentrations normally found in submarine groundwater discharge, SGD may drive nitrate limited coastal and marine systems to phosphate limitation. This is particularly so in karst systems which are normally well oxygenated, resulting in often near-conservative nitrate transport while phosphate is efficiently removed (Kamermans et al. 2002; Slomp and Van Cappellen 2004). Conversely however, SGD has been linked to offsetting P limitation in a karst system via phosphate and/or iron loading (Carruthers et al. 2005). Loading of N and/or P to N and/or P limited systems may favour non-diatom taxa (e.g. dinoflagellates) which have a reduced Si growth requirement, with concomitant shifts in subsequent tiers of the food web (Radach et al. 1990).

Only relatively recently has SGD been recognised by the scientific community as an ecologically significant process and to date there exists a very limited body of work investigating its ecological effects. The presence of SGD has been postulated as a requirement for the maintenance of some habitats, including vertical intertidal zones (Johannes 1980; Kohout and Kolipinski 1967), coral reef systems (Johannes 1980; Paytan et al. 2006) and commercially important macroalgal assemblages (Pongkijvorasin et al. 2009). Diffuse SGD has in some cases been linked to increased primary production (Miller and Ullman 2004; Waska and Kim 2010b; Migné et al. 2011), red-tide development (Lee and Kim 2007), the onset and development of eutrophication (Dong-Woon Hwang et al. 2005; Valiela et al. 1990) and harmful algal blooms (Paerl 1997), and changes to species diversity (Kamermans et al. 2002). SGD has been linked to the altered composition and biomass of seagrass and macroalgal communities (Carruthers et al. 2005; Mutchler et al. 2007; Kamermans et al. 2002), with negative relationships observed between SGD inputs (particularly associated with freshwater) and seagrass diversity, abundance and biomass (Johannes 1980; Kamermans et al. 2002; Mutchler et al. 2007). For example, Valiela et al. (1990) found that diffuse SGD was associated with reduced dominance and growth of eelgrass (*Zostera marina*), greatly increased growth of fast growing opportunistic macroalgae (*Cladophora* and *Gracilaria*) and periodic onset of anoxia, when algal respiration exceeded photosynthesis, with resulting fish and invertebrate mortality. SGD has been associated with alterations to the macroinvertebrate community, with some studies describing increased species diversty, whilst others, decreased species diversity (Migné et al. 2011a; Zipperle and Reise 2005; Kotwicki et al. 2013; Silva et al. 2012; Dale and Miller 2008).

Like surface freshwater estuaries, nutrient inputs via SGD are handled differently by different systems. Attributes of individual subterranean estuaries and associated coastal systems 'filter' the nutrient enrichment via SGD, resulting in varying responses. Some of the attributes or factors which contribute to this 'filtering capacity' are likely to be associated with SGD and specific to SGD relative to surface estuaries, e.g. microbial nitrogen removal in the aquifer.

1.4. Submarine groundwater discharge and karst systems

Submarine groundwater discharge is frequently found where karst aquifers are hydraulically connected to the sea (Fleury et al. 2007; Slomp and Van Cappellen

2004). For exasmple, Karst-channelled SGD has been described in Italy (Moore 2006), Turkey (Bakalowicz et al. 2008), Israel (Swarzenski et al. 2006), Spain (Garcia-Solsona et al. 2010; Mejías et al. 2012), the U.S.A. (Charette et al. 2013), Ireland (Cave and Henry 2011) and Australia (Johannes 1980; Johannes and Hearn 1985). Pathways created by limestone dissolution allow rapid infiltration and relatively unrestricted conduit flow of groundwater in Karst aquifers. These natural pathways provide focused, well-defined coastal entry points rendering karst-channeled SGD a 'point' rather than 'diffuse' mode of delivery of SGD. Large volumes of freshwater SGD can be rapidly delivered to the coast via these point entries (Drew 2008), reducing the marine water dilution capacity in the receiving environment.

Karst aquifers are normally associated with thin soils and subsoils and rapid conduit groundwater flow. This generally results in a short time lag between groundwater pollution events at source (soil leaching, septic tank leakage, etc.) and discharge to the sea. Furthermore, groundwater in karst systems generally interacts closely with surface water bodies (rivers, lakes and turloughs (ephemeral lakes)). These features lend groundwater in karst aquifers to both contamination and contaminant transport more so than other hydrogeological settings (Coxon 2011). These characteristics also minimise opportunities for attenuation via absorption, ion exchange, chemical breakdown, microbial die-off, or removal processes. As a result, agricultural activities on karst catchments which are hydraulically connected to the coastal zone are linked to increased transport of contaminants to the coast. This contamination derives from many sources, both diffuse (organic and synthetic fertilizers) and point (leaking of stored animal waste and septic tank effluent) (Coxon 2011). The pollution type is commonly nutrients (nitrate and phosphate), pesticides and other organic compounds, and microbial pathogens (Coxon 2011). During passage of groundwater through the aquifer, subterranean estuary and beach face sediments, nitrogen attenuation and removal can occur by various processes, particularly denitrification in anoxic environments, lowering groundwater nitrogen concentrations (Rocha et al. 2009). Low aquifer residence time and normally well oxygenated conditions in karst aquifers, however, reduce the capacity for nutrient mitigation and removal during transit (Slomp and Van Cappellen 2004). Thus karst aquifers generally have a higher propensity to transport larger volumes of more nitrogen-enriched freshwater to the coast than other aquifer types which are normally associated with lower water fluxes and reduced nitrogen concentrations (Slomp and Van Cappellen 2004; Coxon 2011). Therefore, in the same receiving environment, karst-channelled point-source SGD is expected to induce more acute ecological changes than those caused by diffuse SGD due to differences in freshwater flux and nutrient (particularly nitrogen) loading associated with each delivery type.

Karst and carbonate systems, which have a higher propensity to transport large fluxes of freshwater SGD and dissolved nitrogen than other hydrogeological setting account for 25 % of the world's coastline (Ford and Williams 2007). Despite this, little work has been done to determine the ecological impacts of karst-channelled SGD.

1.5. Disturbances, ecological succession and SGD

1.5.1. Disturbances

Natural communities may be in a state of global equilibrium. That is, the absolute and relative abundances of species remain relatively constant or undergo regular cyclical oscillation and most species persist for many generations over a large area (Sousa 1979a). This equilibrium is however rarely maintained locally. At the local level, equilibrium is altered by periodic or stochastic disturbances or more gradual changes in the physical environment (Sousa 1979a). Disturbances are generally temporally and spatially heterogeneous, and generate patchiness in natural systems (Sousa 1979b; Levin and Paine 1974). In this way, disturbances renew limiting resources (often space) and promote the local coexistence of species (Sousa 1979b). Migration between patches created by disturbances allows species to persist globally though they may go extinct locally (Hutchinson 1951). Recurrent patch disturbance causes changes and extinctions of local populations of species and is characteristic of most natural systems (Sousa 1979a).

Disturbance in the context of coastal and estuarine systems can be natural, but also anthropogenic. Natural coastal disturbances include the effect of drift material such as logs which batter the intertidal environment (Dayton 1971, p.19), wave action which can open patches in intertidal mussel beds (Levin and Paine 1974), overturning of intertidal boulders which partially or completely kills algae and invertebrates and exposes new surfaces for colonisation (Sousa 1979b), and salinity gradients. Anthropogenic disturbances include, for example, overfishing (Jackson et al. 2001), coastal dredging (Newell et al. 1998), the establishment of fish farms (La Rosa et al. 2001), the release of freshwater (Cañedo-Argüelles and Rieradevall 2010) and anthropogenic nutrient enrichment (Gray and Ying 2002). Eutrophication is deemed one of the most widely occurring and severe disturbances in coastal marine systems (Gray and Ying 2002).

Local species diversity is only maintained when disturbances are sufficient in frequency and intensity to keep the species assemblage in a nonequilibrium state (Sousa 1979a). In the absence of disturbance, species diversity often declines as succession leads to local monopolisation of space and resources by competitively dominant and/or long-lived species. This has been observed in intertidal algal and macroinvertebrate communities (e.g. Lubchenco and Menge 1978 and Dayton 1971, respectively). Areas subject to disturbance with intermediate frequency generally display the highest species diversity, while areas which experience disturbance either comparatively frequently or infrequently generally display lower diversity and are strongly dominated by a small number of species (Sousa 1979b).

1.5.2. Succession

Following a disturbance event, recolonisation by primary producers and sessile species occurs via ecological succession (Connell and Slatyer 1977; Sousa 1979b). Ecological succession is the process of change in the species structure due to species replacement and change in performance of species comprising the ecological community over time (Luken 1990; Pickett et al. 1987; Connell and Slatyer 1977). Following renewal of space and other resources following a disturbance, 'opportunistic' or 'pioneering' species with broad dispersal powers and rapid growth colonise first (Connell and Slatyer 1977). The subsequent sequences in ecological success are many and varied but can be broadly grouped into, or explained in terms of, three models; (1) facilitation; (2) tolerance; and (3) inhibition (Connell and Slatyer 1977). Under the 'facilitation' model, the colonization and growth of subsequent species may be dependent on and facilitated by alterations to the environment by the first colonizing species. Alternatively, under the 'tolerance' model, the successional sequence may be enabled as different species have evolved different mechanisms of resource exploitation and are thus able to tolerate each other's presence. Finally, under the 'inhibition' model, all species resist invasions from competitors. The first colonizing species exclude or inhibit later colonists until they are damaged or die off, thus releasing resources and allowing later colonists to reach maturity (Connell and Slatyer 1977). There is a large body of evidence in support of the 'inhibition' and 'facilitation' models, but less evidence in support of the 'tolerance' model (Connell and Slatyer 1977; Farrell 1991). In reality, ecological succession normally involves more than one mechanism or mode of occurrence (Chapin et al. 1994; Farrell 1991).

1.5.3. Disturbance, succession and diversity

The frequency of physical disturbance strongly affects species diversity and composition by determining the time interval over which successional species replacement can occur (Sousa 1979a). During succession following a disturbance, initially, diversity increases as species colonise bare surfaces but later diversity declines as one species dominates the space (Sousa 1979b). Areas of infrequent disturbance usually display low species diversity as succession results in dominance by late successional species. This is illustrated by the seminal research of Sousa (1979b) who considered marine intertidal boulder fields in southern California. Sousa (1979b) found that when a surface was cleared either by natural disturbance or experimentally, it was first colonized by the fast growing opportunistic green macroalgae, *Ulva* spp. and the barnacle *Chthamalus fissus*. In the latter half of the first year after clearing, diversity increased as several species of perennial algae colonized the surface. In the case of no intervening disturbance, a late successional species gradually dominated, holding 60 to 90% of the space after two to three years and resulting in reduced species diversity (Sousa 1979b). Areas subject to frequent disturbances on the other hand are available for colonization only for short periods of time before the disturbance re-occurs, starting the successional process again (Sousa

1979a). These patches are characteristically continually in the early stages of colonization with relatively large portions of uncolonized space, and low species diversity dominated by species which colonise and develop rapidly at the relevant time year (Sousa 1979a). Areas subject to intermediate frequency of disturbance tend to be the most diverse.

Differences in the ecological composition of neighbouring areas/patches are the direct result of differences in the intensity, periodicity, and exact history of disturbances experienced by each area/patch. Sites in receipt of SGD and control sites which are in close spatial proximity (i.e. adjacent) and at the same shore height are expected to experience the same intensity and periodicity of disturbances other than that associated with SGD. Differences in the community composition between neighbouring SGD and control sites may be related to that which might be predicted by ecological succession following the effect of a disturbance, i.e. SGD. SGD as a form of 'disturbance' will be further explored in the general discussion (Chapter 6) in light of the results of the research herein.

1.6. Salinity based zonation, biotic indices and SGD

Numerous physicochemical parameters of SGD may differ from the receiving environment, including salinity (Hays and Ullman, 2007; De Sieyes et al., 2008; Leote et al., 2008) and macronutrient concentrations (Charette and Buesseler, 2004; de Sieyes et al., 2008; Leote et al., 2008; Garcia-Solsona et al., 2010). These parameters may individually and/or synergistically alter the form and structure of the associated intertidal biotic assemblage. Traditionally, nutrient inputs were generally considered the primary factor affecting macroalgal production and growth rate. Recent studies have however found salinity to be the most important factor conditioning production and growth in coastal systems (Nygård and Dring 2008). Where SGD delivers significant volumes of freshwater as well as nitrogen to the receiving marine environment, it is necessary to determine the influence of the disturbance caused by salinity on ecology, as well as and independent from the influence of the disturbance caused by nitrogen additions on ecology. Two commonly used tools which may be used to help understand the response of ecological communities to SGD are (1) systems which define salinity zones based on associated biology (e.g. Venice System 1959; Bulger et al. 1993), and (2) species diversity indices which help elucidate the effects of disturbances including, and independent of, salinity (Borja et al. 2000; Muxika et al. 2005).

1.6.1. Comparing surface and subterranean estuaries

Freshwater flow is the principal cause of physical variability in surface estuaries and said to 'define' surface estuaries (Kimmerer 2002). SGD may comprise freshwater, but often comprises some portionof seawater, and may comprise completely of recirculated seawater (Nakada et al. 2011; Santos et al. 2012; Leote et al. 2008). Submarine groundwater discharge may differ from surface estuaries in freshwater content (and thus salinity) and/or physicochemistry.

Some instances of SGD may be comparable to surface estuaries (i.e. those containing some part freshwater) by way of decreased salinity. For this reason value can be gained in some cases from comparing SGD with current knowledge of ecological salinity effects in surface estuaries. However, where SGD comprises freshwater and is of similar salinity to a freshwater estuary, the ecological community may be modulated by other environmental gradients (e.g. pH, temperature, turbidity and substrate (Bulger et al. 1993)) which differ between subterranean estuaries (SGD) and surface estuaries. Thus, while it is useful to consider the effects of the altered salinity associated with SGD, when relevant, with reference to those effects documented for surface estuaries, the information gleaned from such comparisons is limited by the large differences in other physicochemical parameters between surface estuaries and SGD. Indeed, where the SGD comprises largely or completely of re-circulated seawater, comparisons with surface estuaries are redundant. The recirculated seawater portion of SGD has salinity equal to or even greater than that of the receiving environment. This recirculated SGD can transport oxygenated seawater, dissolved substances, fine particles, bacteria, viruses and phytoplankton into sediments, and release previously bound nutrients, refractory particles, and organisms from sediments (Santos et al. 2012; Moore 2010; Boehm et al. 2004; Patten et al. 2008; Huettel et al. 2007). Thus, the recirculated seawater component of SGD can significantly alter the biogeochemistry of sediments and overlying waters (Santos et al. 2012).

SGD occurring at some of the study sites in the current research was expected to comprise at least some portion freshwater. Thus the ecology associated with decreased salinities in surface estuaries will be introduced here. The interrelation between the observed ecology and salinity will be incorporated into the subsequent chapter discussions, where relevant. I will start by outlining the typical ecologically relevant salinity ranges/bands by reference to the Venice System (1958). I will proceed to introduce a similar system developed by Bulger et al. (2003), and conclude by outlining some examples which might be comparable to the systems studied herein.

1.6.2. The Venice System

Estuarine species are not evenly distributed across estuarine salinity gradients. Instead, there exist only a few basic patterns of estuarine utilization, allowing grouping of biological structure by salinity zones (Bulger et al. 1993). Estuarine biological zonation can be described based on salinity bands by way of the Venice System where the salinity classification corresponds approximately with biological zonation (Venice System 1959). Within this system, waters with salinity 30 to 40 psu are euhaline; salinity greater than 40 psu are hyperhaline; salinity less than 0.5 are limnetic (freshwater); and salinity 0.5 to 30 psu are mixohaline or brackish, though the term 'mixohaline' is used in preference to 'brackish' (Venice System 1959). The mixohaline bracket can be further divided into polyhaline (18 to 30 psu), mesohaline (5 to 18 psu) and oligohaline (0.5 to 5 psu) (Venice System 1959). Waters of unstable or variable salinity (irrespective of the mean values) may be referred to as "poikilohaline", while those of stable or constant salinity may be described as of "homoiohaline" (Venice System 1959).

As noted in the Venice System of classification, the ecology which develops in a system is not solely a function of salinity, but rather 'other highly varying features of paramount ecological importance' will also condition the form of the associated ecological assemblage (Venice System 1959). These salinity-based zones/classifications may be further subdivided locally, where appropriate, based on ecological observations/patterns of zonation (Venice System 1959). The Venice system (1959) acknowledges that some systems may experience extreme salinity oscillations which prohibit classification into any one particular band, however, reference to the terms contained within the Venice system may at least facilitate meaningful descriptions of such systems and characterisation of the fluctuation range

(Venice System 1959). The Venice system (1959) has been widely used to describe patterns of distribution of estuarine organisms, however the biological basis for the zonation of the Venice System was not reported in the original document beyond the statement that the subdivisions are based on "biological observations" (Venice System 1959). This somewhat limits it to application of salinity bands only without comparisons of assemblages found at the same bands across different systems.

The Venice system (1959) was subsequently built upon, most notably by Bulger et al. (1993), who provided a comparable salinity classification system based on the biological community and included details of the biological communities associated with each band. Bulger et al. (1993) used principal component analysis (PCA) of 316 species/life stages in the mid-Atlantic region (mainly Chesapeake Bay and Delaware Bay) to identify five overlapping biologically relevant salinity zones, (i) 0 - 4 psu, (ii) 2 - 14 psu, (iii) 11 - 18 psu, (iv) 16 - 27 psu and (v) >/= 24 psu.

The oligohaline-mesohaline boundary for the Venice system (Venice System 1959) and Bulger et al. (1993) are similar at 5 and 4 psu, respectively. The biological justification of Bulger et al. (1993) for this boundary is based on the ranges of freshwater fish included in their analysis. The 2 – 14 psu zone is based largely on larval and juvenile fish, and adults of estuarine fish, very euryhaline marine fish and invertebrates with lower limits 1 psu, and euryhaline freshwater fish with upper limits 13 psu (Bulger et al. 1993). Invertebrates were most associated with the 11 to 18 psu zone, as were immature and adult fish (Bulger et al. 1993). Euryhaline marine invertebrates and fish as well as all life stages of estuarine fishes were associated with the 16 to 27 psu zone (Bulger et al. 1993). The upper boundary for zone ii (14 psu), lower boundary for zone iv (16 psu) and upper boundary for zone iii (18 psu) according to the system of Bulger et al. (1993) may be comparable to the Venice System mesohaline-polyhaline bound at 18 psu (Venice System 1959). Thus, by reference to the two systems, the 14 to 18 psu region may present a general important boundary or approximate salinity limit (Bulger et al. 1993).

Unlike the Venice System, the system presented by Bulger et al. (1993) did not identify 30 psu as a threshold of ecological significance. Instead, the final zone identified by Bulger et al. (1993) ranges from 24 psu upwards. The work of Bulger et al. (1993) largely applies to fish species. Due to their restricted movement and therefore great exposure to their surrounding environment, invertebrate species may display more restricted and specific salinity zone associations, tolerances and ranges than fish species. The 24 psu salinity limit described by Bulger et al. (1993) may reflect fish tolerances while the 30 psu threshold proposed by the Venice System (1958) may reflect to a greater extent invertebrate restrictions and requirements. The 2 psu and 30 psu lower and upper salinity thresholds for estuarine species presented by Bulger et al. (1993) are close to the Venice System (1958) suggested comparable boundaries of 0.5 and 30 psu.

Bulger et al. (1993) also recognised that though the direct effects of salinity upon organisms may be responsible for some of the patterns observed, salinity is not the only variable which conditions the distribution of estuarine species. Also important, are for example; temperature, substrate and turbidity (Bulger et al. 1993).

1.6.3. Species diversity metrics

1.6.3.1. Species-abundance-biomass curves

At the most basic level, the response of macrobenthic communities to stress can be measured by changes in univariate metrics, including species richness, total abundance, and total biomass (Pearson and Rosenberg 1978). Benthic communities in fairly constant undisturbed environments tend to display high species numbers and biomass with moderate abundances, and undergo small quantitative and qualitative changes over time (Pearson and Rosenberg 1978). These parameters can be depicted on species-abundance-biomass (SAB) curves which summarise changes in basic faunal parameters occurring (a) along a transect of decreasing disturbance impact at the same time point, or (b) at the same sampling location during time (Pearson and Rosenberg 1978). Application of these curves to variously impacted systems demonstrates that community structure changes differently to stress across different systems (Pearson and Rosenberg 1978). Species-abundance-biomass curves have been developed and extended to abundance biomass comparisons (ABC) plots which rely on comparison of the distribution of numbers of individuals among species with the distribution of biomass among species to detect disturbances and assess community health (Warwick et al. 1987). Both SAB and ABC plots change in configuration as ecological succession takes place (Warwick et al. 1987; Pearson and Rosenberg 1978). Species-area-biomass and ABC curves can be used in tandem with the theory of ecological succession to understand and predict the effects of disturbances on communities. Data on species richness, total abundance, and total biomass underpin SAB and ABC curves, and combined with data on the taxon present these three metrics also underpin species diversity and biotic indices.

1.6.3.2. Species diversity indices

The number of species alone is insufficient for describing the structure of the species assemblage in a given area because the number of individuals per species also varies (Gray 2000). Thus, species diversity includes two aspects, the number of species in a given area (species richness) and number of individuals of each species present or species evenness (Gray 2000; Magurran 2004; Hill 1973). Evenness refers to the departure of the observed pattern from the expected pattern in a hypothetical assemblage where all species are uniform in the number of individuals present (Magurran 2004, p.102). There are many potential indices of species diversity and many reviews of which indices are most appropriate (e.g. Hill 1973; Magurran and Magurran 1988; Magurran 2004; Gray 2000). Of the commonly used indices three in particular are recommended in general, and particularly for application to marine benthic species diversity (Gray 2000). These are (1) total species richness (S); (2) Shannon-Wiener index (H'); and (3) Simpson's index (D).

Species richness is measured simply by summing the number of species found in a given area. There are two main ways of expressing estimates of species richness, as *numerical species richness*, which is the number of species per specified number of individuals or biomass, or *species density*; the number of species per specified collection area or unit (Hutchinson 1959; Magurran 2004, pp.75–76).

The Shannon-Wiener index (H') incorporates the degree of evenness in species abundances and is given by

$$H' = - \Sigma pi (\ln pi)$$

where *pi* is the proportion of individuals found in the *ith* species (Magurran 2004, pp.107–108).

Though the Shannon-Wiener index incorporates the degree of evenness in species abundance, it still emphasizes the species richness component of diversity (Hutchinson 1959; Magurran 2004, p.114). Simpson's index (*D*) on the other hand is weighted by the abundances of the commonest species and emphasizes the dominance as opposed to richness component of diversity (Hutchinson 1959; Magurran 2004). Simpson's index (*D*) is thus sometimes referred to as a dominance or evenness measure (Magurran 2004). Simpson's index is based on the probability of any two individuals drawn from an infinitely large community belonging to the same species and is given by

$$D = \Sigma p_i^2$$

where p_i is the proportion of individuals in the *ith* species. The value of D ranges between 0 and 1, where 0 represents infinite diversity and 1, no diversity. That is, the bigger the value of *D* the lower the diversity. As this is neither logical nor intuitive, *D* is often expressed as 1-*D* or 1/*D* (Magurran 2004, p.115). Both of these latter indices result in a value which is positively proportional to the species richness of the sample.

In the marine environment, measures of species richness and heterogeneity are commonly applied to assess the impact of disturbances (Gray 2000). These indices do not however take into account information provided by taxon, i.e. differences in taxon tolerances/sensitivity to disturbance/pollution stress. The taxon of species present are also of importance when assessing the impact of disturbances in the marine environment. To accommodate this, a number of other biotic indices have been devised which incorporate information about the specific taxon of species present, as well as species richness and abundances.

1.6.3.3. Extended, stress-specific biotic indices

A number of biotic indices have been proposed to assess ecosystem health and thus determine natural and anthropogenic impacts in estuarine and coastal systems (please see Pinto et al. (2009) for a comprehensive list). One such index is the AZTI Marine Biological Index (AMBI), developed by Borja et al. (2000). AMBI was developed to establish the ecological quality of European coasts based on soft-bottom benthos. It has been successfully applied to different geographical areas and different impact sources, with increasing user numbers in Europe (see Muxika et al. (2005)). The AMBI is also used for determination of the European *Water Framework Directive* (WFD) (2000/60/EC) ecological quality status (EcoQ) (Muxika et al. 2005).

AMBI is based on relatively sedentary macrobenthic animals as they cannot avoid deteriorated water/substrate conditions, have relatively long life spans and consist of different species that exhibit different tolerances to stress (Borja et al. 2000). Borja's AMBI index (Borja et al. 2000) builds on the paradigm of Pearson and Rosenberg (Pearson and Rosenberg 1978) that the response of benthic communities to improvements in habitat quality follows three steps; (1) the abundance increases (2) species diversity increases and (3) the dominant species shifts from pollution-tolerant to pollution-sensitive.

The AMBI is determined by calculating a Biotic Coefficient (BC) which is dependent on the proportions of individual abundances in five ecological groups. Over 6,000 taxa have been identified and classified into these five ecological groups to construct the index. The physicochemical parameters of the sediment and overlying water column, the percentage mud within the sediments, and the concentration of dissolved oxygen and pollutants in sediments present environmental gradients which condition the composition of the macro-benthic community (Borja et al. 2000). The proportion of individuals present from each group is related to the degree of sensitivity/tolerance to these environmental stress gradients. Species in ecological Group I are very sensitive to organic enrichment and present under unpolluted conditions. They include specialist carnivores and some deposit-feeding Tubicolous Polychaetes. Species in ecological Group II are indifferent to enrichment, always present at low densities with non-significant variations over time and include suspension feeders, less selective carnivores, and scavengers. Species in ecological Group III are tolerant of excess organic matter enrichment and may occur under normal conditions but their populations are stimulated by organic enrichment. They are surface deposit-feeding species, such as Tubicolous Spionids. Group IV comprises second-order opportunistic species. These are mainly small-sized Polychaete subsurface deposit-feeders, such as Cirratulids. Group V comprises first-order opportunistic deposit feeders which proliferate in reduced sediments (Borja et al. 2000).

The Biotic Coefficient (BC) upon with the AMBI is based is given by:

Biotic Coefficient (BC) = {(0 x % GI) + (1.5 x % GII)+ (3 x % GIII)+ (4.5 x % GIV)+ (6 x % GV)}/100,

where GI to GV indicate the ecological Groups I through V.

The BC can derive a series of continuous values from 0 to 7, with 7 indicating azoic (devoid of life) conditions. The BC is referenced to a Biotic Index (BI), representing the quality of bottom conditions in a discrete range from 0 (unpolluted) to 7 (extremely polluted). This provides a pollution classification of the site which is a function of the BC. See table 1-1 for a summary of the biotic index, associated pollution classification, dominant ecological group and benthic community health.

Table 1-1 Summary of AMBI showing the Biotic Co-efficient (BC), associated Biotic Index (BI), pollution classification, dominant ecological group and benthic community health. Adapted from Bjora et al. (2000).

Pollution Classification	Biotic Co-efficient	Biotic Index	Dominant Ecological Group	Benthic Community Health
Unpolluted	$0.0 < BC \leq 0.02$	0	I	Normal
Unpolluted	$0.2 < BC \leq 0.2$	1		Impoverished
Slightly polluted	$1.2 < BC \leq 3.3$	2	III	Unbalanced
Meanly polluted	$3.3 < BC \leq 4.3$	3		Transitional to pollution
Meanly polluted	$4.5 < BC \leq 5.0$	4	IV-V	Polluted
Heavily polluted	$5.0 < BC \leq 5.5$	5		Transitional to heavy pollution
Heavily polluted	$5.5 < BC \leq 6$	6	V	Heavy pollution
Extremely polluted	$BC \geq 6$	7	Absent	Absent

AMBI is based on a paradigm which emphasizes the influence of organic matter enrichment on benthic communities (Pearson and Rosenberg 1978). It has however proven useful in assessment of other anthropogenic impacts such as physical habitat alterations including dredging, engineering works, nutrient enrichment and heavy metal inputs (Borja et al. 2000; Muxika et al. 2005). The AMBI system has been tested, validated and found to provide a sound tool for assessing ecosystem health (Pinto et al. 2009; Muxika et al. 2005). The results of the AMBI are consistent with those obtained using other methods and parameters, such as richness, diversity and evenness (Borja et al. 2003). Using the AMBI, communities act as ecological indicators of the 'health' of a system, and can clearly indicate the gradient of disturbance (Muxika et al. 2005). As AMBI is based upon general ecological principles and paradigms, which are independent of longitude and latitude, it is appropriate for use in all European coastal environments (Muxika et al. 2005).

1.6.3.4. Indices, salinity and submarine groundwater discharge

Previous analysis of indices and the effects of salinity on benthic invertebrates has indicated a positive relationship between salinity and number of species (see for example Zettler et al. (2007)). The salinity gradient acts as a natural stressor affecting benthic diversity in a similar way to a human impact (Zettler et al. 2007). This correlation between salinity and species number disappears at salinities > ~15 psu (Zettler et al. 2007). This benthic macroinvertebrate based salinity boundary corresponds well with the 14 - 18 psu boundary suggested by the salinity zonation proposed by the Venice System (Venice System 1959) and Bulger et al. (1993). In areas of strong salinity gradients, the ecological quality/health classification based on macroinvertebrate/macrozoobenthic communities as an indicator depends strongly on the biotic index used as they are variously influenced by salinity (Zettler et al. 2007).

The Shannon-Wiener index for example is strongly influenced by and correlated with salinity (Zettler et al. 2007). This is because this index is strongly influenced by species richness and dominance (Zettler et al. 2007). Where the environmental disturbance under consideration is not salinity, reduced salinity may confound the results if the Shannon-Wiener is employed for reduced salinity environments. AMBI on the other hand is only weakly correlated with salinity (Zettler et al. 2007), making it a more appropriate index in areas of reduced and/or alternating salinities. A modified version of the AMBI, the Multivariate-AMBI (M-AMBI) (Muxika et al., 2006), is a proposed superior metric in waters with a strong salinity gradient (Zettler et al. 2007). M-AMBI is based on benthic macroinvertebrate composition. It is a multivariate combination of AMBI, richness and diversity, factor analysis and discriminant analysis (Muxika et al. 2007).

Thus, in the context of SGD, total species richness (*S*); (2) Shannon-Wiener index (*H'*); and (3) Simpson's index (*D*) indices will be applied where the salinity is > ~14 to 18 psu; AMBI and M-AMBI will be applied where the salinity is less than the 14 – 18 psu boundary, and particularly in the 0 - 5 psu band. Application of metrics and indices according to these salinity parameters will allow the effects on ecological health due to non-salinity related parameters of SGD to be deconvoluted from those associated with reduced salinity. Reduced salinity is a natural disturbance while SGD may, in cases at least, present an anthropogenic disturbance, for example where it is associated with loading of anthropogenic nitrogen. Very large and unstable salinity regimes (i.e. poikilohaline (Venice System 1959)) will prohibit assignment to a discrete salinity band/zone. In such cases, other non-salinity based multivariate methods such as non-metric multidimensional scaling (nmMDS) may be usefully applied.

1.7. Limitations when studying the ecological effects of SGD

1.7.1. SGD reference conditions

The most important issue within the WFD is the comparison of assemblages against those found in reference conditions (Muxika et al. 2005; Zettler et al. 2007; Borja 2004). The reference condition for a water body type is a description of the physicochemical elements which corresponds totally or nearly totally to undisturbed conditions i.e. with no, or with only a very minor impact, from human activities (WFD, 2000/60/EC). The WFD identifies four options for deriving reference conditions: (i) comparison with an existing undisturbed site or a site with only very minor disturbance; (ii) historical data and information; (iii) models; or (iv) expert judgment (WFD, 2000/60/EC; Annex II, 1.3(iii)). Reference conditions are ideally defined/described from data (i) which is acquired from multiple sites with similar physical characteristics, from the same ecoregion and habitat type; (ii) that ideally represent minimally impaired or undisturbed conditions; and (iii) that provide an estimate of the variability in biological communities and habitat quality due to natural physical and climatic factors (Borja et al. 2012a)

SGD is a relatively newly characterised process in scientific terms. Only in the last 15 years has it received significant attention, particularly in terms of marine ecological processes (see comprehensive reviews by Moore 2010; Burnett et al. 2003). Due to this, the often relatively localised nature of SGD, and the high spatial and temporal variability of its occurrence (Moore 2010; Burnett et al. 2001), surface water bodies produced by SGD are not explicitly considered under the current Water Framework Directive (WFD). Subsequently, there is no established reference condition for these water body types. Within the WFD, there are however well established reference conditions for estuarine systems. Comparison of SGD sites to conventional surface estuary reference conditions on the basis of similar salinities may provide some useful information. A large amount of caution should be used however when attempting to establish potential reference conditions for SGD systems by comparison with surface estuary reference conditions due to the many and varied documented difference between surface and groundwater estuaries (previously discussed). Though surface estuaries represent the most similar conditions relative to subterranean estuaries and

SGD, the usefulness of comparisons of SGD systems with estuarine reference conditions will be largely restricted to the influence of altered salinity over the biological assemblage, without providing useful information on the influence over ecology of the other factors which may vary in association with SGD (previously discussed).

The most feasible, informative and currently used method for establishing reference conditions and deriving information regarding the ecological effects of SGD is through comparison with closely located (i.e. same ecoregion and habitat) control sites which receive minimal exposure to SGD (Kotwicki et al. 2013; Encarnacão et al. 2013; Silva et al. 2012; Migné et al. 2011b; Ouisse et al. 2011; Miller and Ullman 2004; Dale and Miller 2008; Carruthers et al. 2005). These serve as reference sites. Reference conditions using pristine areas or least disturbed areas is this the preferred method recommended by the European WFD (Borja et al. 2012). It is however recognised that pristine marine and estuarine habitats are rare (Borja et al. 2012). In the case of SGD research in particular, it is acknowledged that these 'reference sites' are relative reference sites for comparison with the effects of SGD only. These reference sites will in most cases still receive some impact from SGD, however, it is assumed that they are the 'least disturbed' areas in terms of SGD, still located in the same ecoregion and same habitat as the SGD site. These sites are picked as they are deemed to have acceptable level of disturbance to represent an achievable or existing reference conditions (Borja et al. 2012). It is acknowledged that such reference conditions do not equate to totally undisturbed, pristine conditions, but include a level of pressure where there are ideally no or only very minor ecological effects from the disturbance under examination, as per Borja et al. (2012). In the current case, the disturbance in question is not reduced salinity, as this is a natural disturbance. The disturbance is the other physicochemical parameters associated with SGD. Thus, the 'relative controls' or 'relative reference' sites in the current study are assumed to experience some impact of SGD, however, the exposure time to SGD is less and the concentration of SGD to which they are exposed is significantly less than that of the site of SGD. Throughout the following document, these 'relative control' sites are referred to as 'control' sites, unless otherwise stated. The sites which receive the greatest exposure to SGD at the greatest concentrations SGD, and thus are of interest in terms of the effects of SGD are referred to as 'SGD' sites, unless otherwise stated.

1.7.2. Methods and techniques in SGD ecological research

The accurate detection and quantification of anthropogenic inputs and ecological impacts associated with SGD is not a straightforward task. Some of the complications include (1) rapid dilution of freshwater inputs by marine sources that can blur the contribution of SGD and cause diffuse, dispersed ecological impacts which may be difficult to distinguish from background noise (Mutchler et al. 2007); (2) conversely, the impact of SGD, both chemical and ecological, may be heightened in smaller bodies of water such as embayments or lagoons due to their limited volume and restricted fluid exchange with the open ocean (Burnett et al. 2003); (3) small scale point sources of SGD may go undetected in sampling and may result in very localised ecological alterations which may be missed if random sampling is applied at a larger scale; and (4) nutrients may be delivered as short-term pulses resulting in a 'pulsed' ecological impact, which again may go undetected during periodic sampling (Mutchler et al. 2007). There are also a number of issues relating to the spatial and temporal variability inherent in SGD and the application of techniques to study the ecological effects of SGD which are suitable despite the aforementioned caveats.

The occurrence of SGD can be spatially and temporally variable, rendering it difficult to locate, identify and characterise. For example, in Ireland alone, over 35 sites of freshwater submarine groundwater discharge are postulated based on geology (Wilson and Rocha 2012), however, only two of these have been verified (Drew and Daly 1993; Wilson and Rocha 2012). Due to temporal and spatial variability, the ecological alterations associated with SGD nutrient loading may not be as pronounced as for other more sustained sources of nutrient loading; however, the ecological alterations may be progressive over time due to cumulative effects of nutrient loading. Such gradual ecological alterations may not be readily perceptible to the naked eye or detectible during periodic routine sampling. Karst systems are particularly relevant in this context as the freshwater flow rate of karst-channeled SGD is often tightly coupled to aquifer recharge rates (mainly via precipitation), so that karst-channeled SGD may be particularly variable in space and time.

As previously discussed, numerous physicochemical parameters of SGD, both freshwater and recirculated seawater, may differ relative to both surface freshwater and marine water. Whilst traditionally nutrient inputs were generally considered the primary factor affecting macroalgal production and growth rate, recent studies have found instead that salinity is the most important factor conditioning production and growth in coastal systems, followed by differences in DIC and nutrient concentrations (Nygård and Dring 2008). The deleterious effects of reduced salinity on the growth of marine macroalgae can however be offset by nutrient additions (Kamer and Fong 2001; Nygård and Dring 2008). This example illustrates the complexity of the effects of altered parameters of physicochemical properties and underpins their relevance as complicating factors when studying the ecological effects of SGD. The ecological effects of SGD nutrient additions may be modulated, ameliorated or exacerbated, by other physicochemical properties of SGD.

The dearth of data and research on the interrelation between coastal food webs and SGD, particularly its borne nutrients, derives not so much from the aforementioned caveats, but more from the absence of suitable techniques to detect SGD, and associate it with food web utilization of SGD-borne nutrients, given of the aforementioned caveats. The technologies and techniques currently used in the study of SGD are; seepage meters; geochemical tracers; hydrogeological models (McCoy and Corbett 2009); isotopes (mostly radioactive isotopes (see, for example, Burnett and Dulaiova 2003; Charette 2007; Moore 2006; Santos et al. 2010) and also though to a far lesser extent stable isotopes (e.g. Carruthers et al. 2005; Mutchler et al. 2007; Ouisse et al. 2011)); and remote sensing of thermal infrared imagery (Wilson and Rocha 2012). These techniques provide quantitative and in some cases qualitative measures of SGD water and nutrient fluxes, but not the associated ecological alterations. Thus, while a number of issues including the physicochemical properties of SGD may complicate the study of the ecological effects of SGD, it is a lack of appropriate techniques which largely precludes research in this field.

1.8. Stable Isotope Analysis (SIA) in SGD research

One technique which may potentially prove useful in addressing the issues inherent in studying SGD ecological impacts is stable isotope analysis (SIA). Isotopes are atoms of the same element which have the same number of protons and electrons but a different number of neutrons in the nucleus (Sulzman 2007, p.1). Isotopes may be energetically stable (i.e. stable isotopes) or unstable (i.e. radioactive). As isotopes of the same element have the same electron valance, their chemical behaviour is qualitatively similar i.e. they have the same propensity to form chemical bonds (Sulzman 2007, p.6). However, due to properties associated with kinetic energy and vibrational energy, the physical behaviour of different isotopes of the same element is qualitatively different leading to differences in reaction rate and bond strength (Sulzman 2007, p.6). Kinetic energy is constant for a given element in fixed environmental conditions. As molecules of the same element in the same physical environment have the same kinetic energy, molecules of larger masses travel at slower velocities (Sulzman 2007, p.7). Thus, atoms of the same element which have different atomic masses react at different velocities, with heavier molecules reacting more slowly (Sulzman 2007, p.7). The frequency of vibration of a molecule determines its vibrational energy. Heavy atoms vibrate more slowly than lighter ones causing molecules containing heavier isotopes to have lower energy allowing them to form stronger, more stable bonds (Sulzman 2007, p.7). As more energy is required to break the bonds formed by heavy-isotope-containing molecules, the rate of chemical reaction of lighter isotopes is greater than that of their heavier counterparts. In terms of reaction kinetics, this implies that over the same period of time, a greater number of lighter isotopes will be involved in reactions. Progressive enrichment of the reaction product in the lighter isotope and unreacted product in the heavier isotope causes their isotopic signatures to diverge both from each other and the original pre-reaction substrate pool. This differential incorporation of the two isotopes along the process path due to the difference in energy required to break bonds results in/is called ‘isotopic fractionation’.

Isotopic composition, denoted δ (delta), is reported as deviation from an internationally accepted standard. Delta values are not absolute isotope abundances but differences between sample readings and the respective standard where all standards have $\delta = 0$.

The analysis of natural abundance stable isotope ratios of carbon ($^{13}C/^{12}C$) and nitrogen ($^{15}N/^{14}N$) is commonly used in many fields of research, including marine ecology and pollution. Nitrogen isotopic signatures are reported relative to the nitrogen standard, atmospheric nitrogen (N_2), and carbon isotopic signatures relative to the carbon standard, Vienna PeeDee Belminite Limestone (vPDB). As isotopic differences between various materials are very small they are expressed in parts per thousand or permil, denoted ‰. Absolute isotope ratios (R) are measured for the sample and international standard, and the relative difference (*δ*) calculated. Thus any isotope signature is calculated and expressed as;

$$((R_{sample} - R_{standard})/R_{standard}) \times (1000\ \delta‰)$$

where R is the ratio of heavy to light isotope, R_{sample} is the sample ratio and $R_{standard}$ that ratio in the standard (Sulzman 2007, p.6). Samples with a positive delta (*δ*) have more of the heavy isotope than the standard and vice versa (Sulzman 2007, p.6). This is the 'isotopic signature' of that element in the molecule pool in question.

The utility of stable isotopes is based on the premise that the isotopic signature of nitrogen and carbon at the base of the food web is indicative of the origin of the nutrients and when these nutrients enter the food web, their isotopic signatures change in relatively predictable or at least traceable ways (DeNiro and Epstein 1978; DeNiro and Epstein 1981; Zanden and Rasmussen 2001). Analysis of these isotopes has permitted investigation of food web structure (Couch 1989; Layman et al. 2007) and links between trophic levels (Post 2002), as well as investigation of the nitrogen and carbon sources at the base of food webs (Currin et al. 1995; McClelland et al. 1997).

SIA may be particularly useful in SGD research for two reasons. Firstly, different nitrogen sources display characteristic isotopic signatures (Figure 1-3), sometimes allowing investigators to identify anthropogenic sources of nitrogen in groundwater, where they exist (Kendall and Aravena 2000; Xue et al. 2009). This may enable determination of sources of SGD-borne nitrogen, particularly anthropogenic N. Also, DIC in freshwater is depleted in ^{13}C relative to marine DIC. This feature allows discernment of freshwater dependent food webs from marine water dependent food webs. This attribute is particularly useful for studying the ecology associated with freshwater SGD.

The isotopic signatures of N and C (expressed as $\delta^{13}C$ and $\delta^{15}N$ respectively), change in relatively systematic ways as they enter and move through the food web. The N and C isotopic signatures of primary producers reflect that of the C and N nutrient sources with fractionation proportional to the excess of nutrient supply relative to demand (Marshall et al. 2007, p.36; Mariotti et al. 1981; Kendall et al. 2007, p.393; Raven et al. 2002; Maberly et al. 1992). Herbivore carbon isotope ratios are enriched in ^{13}C relative to their primary producer source by ~0 to +1 ‰ (DeNiro and Epstein 1978; Peterson and Fry 1987), and similarly, nitrogen isotope ratios are generally enriched in ^{15}N by +2.5 to +3.4 ‰ (Zanden and Rasmussen 2001; Vanderklift and Ponsard 2003; DeNiro and Epstein 1981). These features permit investigation of food web N and C dynamics (Currin et al. 1995; McClelland et al. 1997) and allow researchers to trace the trophic transfer of these nutrients of known origin into and through food webs.

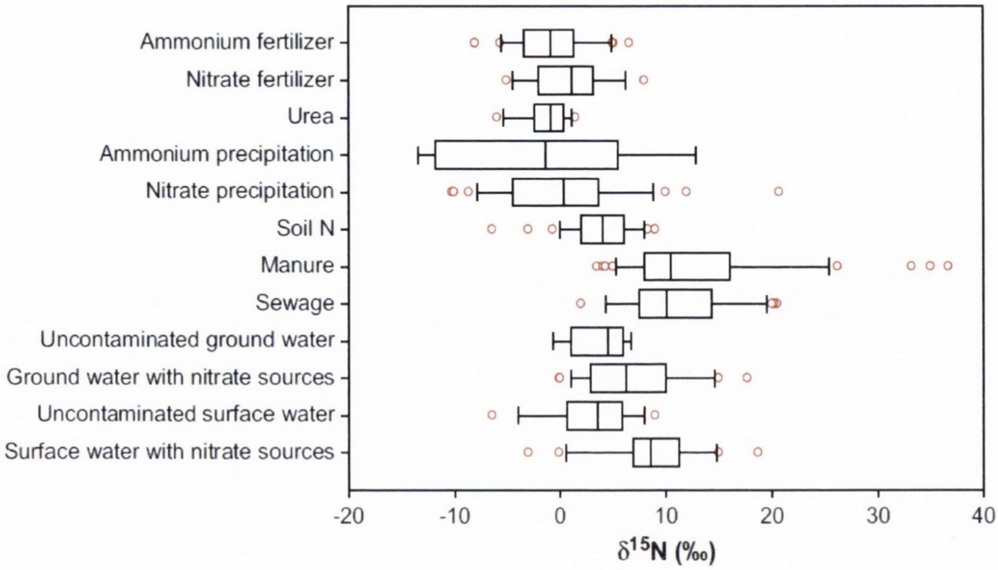


Figure 1-3 Box plots of $\delta^{15}N$ values of NO_3^- from various sources and sinks. Box plots illustrate the 25th, 50th and 75th percentiles; the whiskers indicate the 10th and 90th percentiles; and the circles represent outliers. Compiled and adapted from a comprehensive literature review by Xue et al. (2009).

Though stable isotope analysis (SIA) of organism tissue is commonly used in ecological and pollution research, it has been employed in only a small number of SGD studies (Kamermans et al., 2002; Carruthers et al., 2005; Mutchler et al., 2007; Ouisse et al., 2011). Of the four previous studies of SGD which used SIA of organism tissue, three considered algal/seagrass $\delta^{15}N$ only (Kamermans et al. 2002; Carruthers et al. 2005; Mutchler et al. 2007), while one considered sediment organic matter and macroinvertebrate $\delta^{13}C$ and $\delta^{15}N$ (Ouisse et al. 2011). Three of these studies investigated at the effect of SGD-borne wastewater on select species of primary producers (Kamermans et al. 2002; Carruthers et al. 2005; Mutchler et al. 2007) and the fourth considered the effect of SGD on food web structure (Ouisse et al. 2011). While these studies provide interesting results, none investigated the utility of SIA in (1) identifying food webs in receipt of SGD and delineating SGD from control food webs, or (2) tracing the trophic transfer of SGD-borne N and C into and through coastal food webs, where the SGD has not been subject to a discrete, significant pollution event.

In combination with conventional ecological surveys and water chemistry and nutrient analysis, SIA may address the current methodological deficit in SGD ecological research. SGD may facilitate assignment of SGD-borne nutrients to their ecological and functional ecosystem impact(s), and, where relevant, source(s). If methods can be standardised, SIA may provide a robust, informative tool facilitating recommendations to coastal managers regarding all aspects SGD including potential mitigating strategies, and eventually allowing imposition of punitive measures leading to enforcement of the 'polluter should pay' principle (97/11/EC). Knowledge of the effect of SGD on the carbon and nitrogen isotopic signatures of food web components might improve our understanding of nutrient use and transfer through SGD food webs, and the theoretical framework underpinning the application of stable SIA in intertidal ecological research.

1.9. Research questions and hypotheses

This research aimed to address two questions. The main question was, does SGD cause direct ecological impacts in intertidal marine environments, and if so, can these impacts be identified and their type and magnitude quantified? Secondarily, can SIA provide an informative technique in the field of SGD ecological research, and if so, what types of information can be gleaned through its use?

To address these research questions, the following alternative hypotheses (Ha) were postulated and associated null hypothesis (Ho) tested:

(1) Ha: The community composition and biomass of macroalgae will differ between SGD and control sites.

Ho: There will be no difference in the community composition and biomass of harvested macroalgae at the SGD and control sites.

(2) Ha: The structure and composition of the non-sessile (i.e. motile) macroinvertebrate assemblage will differ between SGD and control sites.

Ho: There will be no difference in the structure and composition of the motile macroinvertebrate assemblage at the SGD and control sites.

(3) Ha: The community structure and composition, assessed in terms of percentage cover of sessile/attached species (i.e. macroalgae and attached macroinvertebrates), will differ between SGD and control sites.

Ho: There will be no difference in the community structure and composition, assessed in terms of percentage cover of non-motile/attached species (i.e. macroalgae and attached macroinvertebrates), between SGD and control sites.

(4) Ha: There will be a relationship/correlation between the fraction/proportion of freshwater SGD and the observed ecological alterations.

Ho: There will be no relationship/correlation between observed ecological alterations and the fraction/proportion of freshwater SGD.

(5) Ha: The nitrogen isotopic signature of SGD food web components will differ from that of their control food web counterparts.

Ho: The nitrogen isotopic signature of SGD food web components will not differ from that of their control food web counterparts.

(6) Ha: The carbon isotopic signature of SGD food web components will differ from that of their control food web counterparts.

Ho: The carbon isotopic signature of SGD food web components will not differ from that of their control food web counterparts.

To test these null hypotheses, four experiments were conducted encompassing three system types (coastal lagoon, sheltered bay, unenclosed relatively exposed coastline) across two countries representing different ecoregions. These experiments are presented herein as chapters, styled in the format of research articles.

Chapter 2 examines the relationship between intertidal SGD and the structure, form, composition and trophic nutrient transfer of intertidal food webs on a sandy beach face in southern Portugal. This experiment includes the first application of SIA in studying the ecological effects of SGD. SGD is discussed in the context of a disturbance in the system. The null hypotheses (1), (2) (5) and (6) above are tested in Chapter 2.

Chapter 3 considers the effect of intertidal freshwater karst-channeled SGD on the structure and composition of the macroalgal and macroinvertebrate intertidal community both seasonally and relative to control sites, on the Irish west coast. The null hypotheses (1) and (3) above are tested. Furthermore, a discussion is presented on the physicochemical parameters of SGD (nitrogen concentration, salinity and temperature), and on SGD as a disturbance in the system.

Chapter 4 tests null hypotheses (3) and (4) above, addressing the effect of karst-channeled intertidal SGD on the structure and composition of the sessile (macroalgae and attached macroinvertebrate) community in south Portugal, a contrasting ecoregion to the one previously explored on the Irish west coast. The results of the latter two studies are then compared and contrasted and generalisations regarding freshwater SGD in general and karst-channeled SGD in particular suggested.

Finally, Chapter 5 tests null hypothesis (5) and (6) above and more fully explores the utility of SIA in studying SGD. This chapter focuses on karst-channeled freshwater intertidal SGD in Ireland and considers the relevance of altered values of physicochemical parameters associated with SGD.

Chapter 2. Impacts of submarine groundwater discharge on the structure, composition, and nutrient transfer pathways through a lagoonal beach face macroalgal and macroinvertebrate assemblage.

2.1. Abstract

Submarine groundwater discharge (SGD) is ubiquitous in intertidal regions worldwide. The significance of SGD as a structuring agent of the benthic intertidal food web has, however, not yet been considered in ecological studies. Assessing the effect of SGD on the C and N isotopic signatures of food web components might be crucial to improving our understanding of nutrient use and transfer through food webs in areas in receipt of SGD, and to the theoretical framework underpinning the application of stable isotope analysis (SIA) in intertidal ecological research. A field experiment was conducted to determine the effect of SGD on food web composition and structure on a sandy beach face in a coastal lagoon (Ria Formosa, southwestern Iberia). Benthic macroalgae and its related epimacrofauna (> 0.5 mm) were sampled from two areas where SGD was occurring (SGD areas) and two control areas. For each SGD area, the associated control area was devoid of SGD but in close proximity (~100 m) to the control area to ensure similarity of physical conditions. Thus there were two sections along the beach face, beach face section A and beach face section B, both of which contained a SGD and a control area distanced ~100 m apart. Beach face section A and section B were located ~200 m apart. ANOSIM identified an effect of SGD (i.e. assemblages differed due to the presence of SGD regardless of the beach face section) and of the blocking factor beach face section (i.e. assemblages differed between beach face sections regardless of the effect of SGD). Food web structure and composition differed significantly between SGD and control areas. The algal assemblage differed between SGD and control areas ($p = 0.003$) with increased algal species diversity and biomass at SGD areas. SGD was also associated with greater macroinvertebrate abundance ($p = 0.002$) and biomass, though non-significant ($p = 0.06$). To account for the effect of the blocking factor 'beach face section', univariate comparisons including those of $\delta^{13}C$ and $\delta^{15}N$ of food web components were restricted to samples from the same beach face section. Two species, positioned at distinct trophic levels (*Ulva* spp. macroalgae and the infaunal detritivore *Bittium reticulatum*) were depleted in ^{15}N ($p = 0.01$ for algae and $p = 0.04$ for *B. reticulatum*) and enriched in ^{13}C ($p < 0.001$ for algae) at the SGD areas. However, not all results of isotopic comparisons were statistically significant. The results suggest that (a) the presence of SGD resulted in the development of two discrete food webs at the small/medium spatial scale (~100 m), (b) fresh groundwater enriched with nitrogen which is depleted in ^{15}N comprised a small fraction of the SGD resulting in the ^{15}N depleted signature of macroalgae and *B. reticulatum*, and (c) SGD enhanced the rate of carbon turn over and primary producer respiration at control areas resulting in macroalgal ^{13}C enrichment. SGD is therefore a major modulating agent for the structure, composition and development of the benthic intertidal sand flat food web.

2.2. Introduction

Coastal marine ecosystems are affected by a wide range of stressors, both natural and anthropogenic. Extensive research has characterised the impacts of many of these stressors, particularly coastal pollution (e.g. Turner and Rabalais 1994; Paerl 1997; Atalah and Crowe 2010). Similarly, a large volume of work has been conducted to characterise the physical factors considered determinants of coastal benthic intertidal community structure and composition, e.g. waves, currents and tides (Koch 2001), climate (Livingston 1984), sediment grain size (McLachlan 1996; Herman et al. 2001; Koch 2001), local hydrodynamics (Hurd 2000), light penetration (Koch 2001), topographic heterogeneity (Archambault and Bourget 1996). Though submarine groundwater discharge (SGD) is a widespread phenomenon which may act as both a stressor or disturbance and physical determinant of community coastal and intertidal structure and composition, it is comparatively understudied.

SGD is a natural phenomenon, driven by tidal oscillation, positive hydraulic head of terrestrial groundwater, or a combination of both. SGD becomes of ecological and thus human concern when it is associated with high solute (including anthropogenic contaminants) loads or its freshwater component is significantly affected by anthropogenic activities, e.g. extraction. The fresh groundwater component of SGD has been suggested as a driver of potentially important ecological alterations in coastal communities (Taniguchi 2002; Moore 2010). To date, however, the majority of SGD research has focused on detecting and quantifying SGD associated fluxes of freshwater and dissolved/colloidal constituents into the coastal zone. The ecological alterations associated with SGD and its relevance as a factor in determining food web structure have received little attention and remain largely un-quantified (Moore 2010). That research which does exist originates from a small number of geographic locations (McClelland and Valiela 1998; Corbett et al. 2000; Kamermans et al. 2002; Zipperle and Reise 2005; Mutchler et al. 2007; Migné et al. 2011; Ouisse et al. 2011; Silva et al. 2012; Kotwicki et al., 2013), and sometimes focuses on a known significant pollution event conveyed via SGD (Carruthers et al. 2005; Lee et al. 2009).

Where SGD is associated with ecological alterations, this impact might also manifest as shifts in the isotopic signatures of the macronutrients carbon and nitrogen in associated local food webs. If so, this may enable the distinction between food webs at sites of discharge and control food webs. This may also have consequences for the potential application of stable isotope analysis (SIA) in food web studies in areas in receipt of SGD. SIA is commonly used in ecological and pollution research, including a small number of SGD studies (Kamermans et al. 2002; Carruthers et al. 2005; Mutchler et al. 2007; Ouisse et al. 2011). The isotopic signatures of nitrogen and carbon change in relatively predictable ways upon entering a food web. Per trophic level increase, organisms become enriched in ^{13}C by ~0 to 1 ‰ (DeNiro and Epstein 1978; Peterson and Fry 1987) and enriched in ^{15}N by ~2.5 to 3.4 ‰ (DeNiro and Epstein 1981; Zanden and Rasmussen 1999; Post 2002). Analysis of these isotopes permits investigation of food web structure (Couch 1989; Layman et al. 2007), links between trophic levels (Post 2002), and N and C sources at the base of the food web (Currin et al. 1995; McClelland et al. 1997). There is a potential but, as yet, largely unexploited application for SIA in such facets of SGD ecological research. Currently, however, there is a gap in the knowledge regarding the impact of SGD on N and C nutrient transfer through food webs.

This chapter reports on the results of a double faceted experiment conducted on a benthic intertidal food web at a sandy, sheltered beach face in a region which experiences a warm Mediterranean climate (Ria Formosa lagoon, Portugal). Previous research has examined numerous aspects of the SGD in this system, including seepage rate (Leote et al. 2008), associated nutrient fluxes (N species, P and Si) (Leote et al. 2008; Rocha et al. 2009), the role of tide in modulating the timing and magnitude of seepage; effect of SGD-delivered nitrate on benthic nitrogen biogeochemistry (Rocha et al. 2009) and the role of beach sediments in mitigating nutrient nitrate loads on a seasonal scale (Ibánhez et al. 2012). However, to date, research has not been conducted to elucidate the ecological role of SGD in the system. Hence, the first part of this experiment was conducted to assess the role of SGD in determining food web structure and composition. The second experiment investigated if SGD was associated with alterations to the nitrogen and carbon isotopic signatures of food web components. To address these aims, the following null hypotheses (Ho) were tested and alternative hypothesis proposed (Ha):

(1)

Ha: The community composition and biomass of harvested macroalgae will differ between SGD areas and control areas.

Ho: There will be no difference in the composition and biomass of the harvested macroalgae community between the SGD areas and control areas.

(2)

Ha: The structure and composition of the non-sessile (i.e. motile) macroinvertebrate assemblage will differ between SGD areas and control areas.

Ho: There will be no difference in the structure and composition of the non-sessile (i.e. motile) macroinvertebrate assemblages between SGD areas and control areas.

(3)

Ha: The nitrogen isotopic signature of SGD food web components will differ from that of their control food web counterparts.

Ho: The nitrogen isotopic signature of SGD food web components will not differ from that of their control food web counterparts.

(4)

Ha: The carbon isotopic signature of SGD food web components will differ from that of their control food web counterparts.

Ho: The carbon isotopic signature of SGD food web components will not differ from that of their control food web counterparts.

2.3. Materials and methods

2.3.1. Study area description

Located in southern Portugal (26° 58' to 37° 03' N, and 007° 32' to 008° 02' W), the Ria Formosa is a large lagoon (55 km long, 6 km at its widest point), with a surface area of ~111 km^2, consisting of two peninsulas and five barrier islands (Figure 2-1.). The tidal regime is semi-diurnal and mesotidal with an overall average depth of 2 m, average spring tide range of 2.8 m and average neap tide range of 1.3 m. During spring tide around 86 km^2 of the surface area is overlain by water at maximum tidal height, however only about 14 % of this surface is permanently flooded and ~80 % of the bottom sediments are exposed at the spring tidal minimum creating an extensive intertidal zone (Andrade et al. 2004). Water column temperature ranges from 12 °C to 27 °C with an average year round salinity of 35.5 – 36.9 psu (Ferreira 2003). Annual average precipitation at Faro city, which lies on the bank of the central arm of the lagoon, is 480 mm and effective precipitation (i.e. deducting losses via evaporation) for the surrounding 740 km^2 watershed is ~1.2 x 10^6 m^3 (Salles 2001).

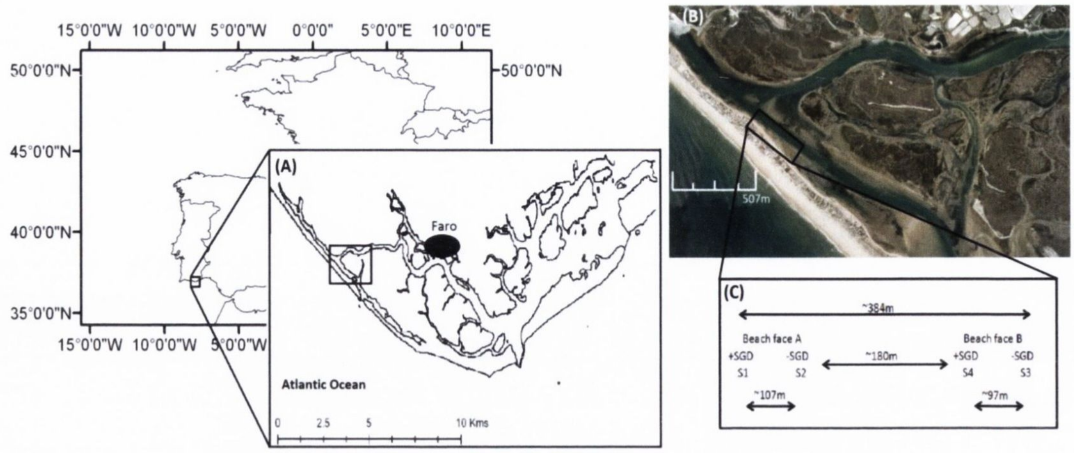


Figure 2-1 The Portuguese location of the Ria Formosa lagoon field site and sampling locations, showing the Ria Formosa lagoon (A), the intertidal location of all four sampling areas (Google Earth 2007a) (B) and the relative locations of the two sections of beach face (beach face A and beach face B) containing four sampling areas (S1 and S2, and S3 and S4 respectively) from each of which five replicate samples were harvested (C).

The lagoon provides a variety of ecosystem services and goods and is of local and regional social, economic and environmental importance (Cristina et al. 2006). Recognition of the importance of the habitat provided by the lagoon has resulted in its protection by a variety of national and international policies (Protection of Habitats and Birds Directive (79/409/EC), Special Area of Conservation (Special Area of Conservation under the EC habitats directive (92/42/EC), and RAMSAR site for protection of wetlands (Cristina et al. 2006)). The lagoon, which is surrounded by an intensively farmed agricultural hinterland, underpins a thriving tourism sector, and in recent years a declining aquaculture sector. Substantial economic development around the lagoon has been associated with deteriorated water quality (Bebianno 1995; Newton et al. 2003). The lagoon is under threat from eutrophication with numerous studies conducted in the past decade to elicit the exact causes of this threat and their relative contribution. Previous research has found that freshwater SGD provides a pathway into the lagoon which delivers nitrate from polluted coastal aquifers (Leote et al. 2008; Rocha et al. 2009).

The food web is primarily based on detritivory (Sprung 1994; Almeida et al. 2008). Planktonic production is relatively low with benthic macrophyte production providing the primary carbon source in the form of detritus (Sprung 1994). Annual macroalgal blooms provide a major source of detritus (Sprung 1994). Most of the food web biomass is contained in the first two trophic levels (producers and detritivores), and to a far lesser extent herbivores (Gamito and Erzini 2005). There are three main intertidal benthic habitat types: sand flats, mudflats and seagrass (*Zostera*) beds (Sprung 1994). At least 53 species occur in the sand flat habitat (see Sprung (1994) for a detailed species list). The most common phylum present in the intertidal benthic food web is Mollusca, with *Bittium reticulatum*, an infaunal detritivorous gastropod, the most abundant species (Sprung 1994; Almeida et al. 2008).

2.3.2. Experimental design and sampling

A blocked test-control sampling design was used to compare SGD areas with control areas. An ecological survey was conducted to test null hypothesis (1) and (2). The number and biomass of algal species was determined at each site and compared using multivariate analysis to test null hypothesis (1). To address null hypothesis (2), the number of species, and abundance and biomass of macroinvertebrate species were determined and subject to multivariate comparisons. A number of diversity indices (species richness (S), Simpsons (D) and Shannon-Wiener (H')) were also computed to elucidate the ecological effects of SGD. Previous research of SGD in the lagoon found that SGD salinity ranged between 17 and 35 psu (Leote et al. 2008). Thus, species richness (S), Simpsons (D) and Shannon-Wiener (H') indices were deemed appropriate for this salinity band (> ~14 – 18 psu). Stable isotope analysis was conducted on organisms found at the SGD and control areas in order to test null hypothesis (3). Furthermore, N and C elemental concentrations of organism tissue were calculated using dry weight, and molar C:N ratios determined to further elucidate the nutrient dynamics at SGD and control sites.

Two sections/blocks of beach face (A and B) were sampled with a SGD and control area in each section, and five replicates at each of the four areas. S1 was the SGD area and S2 the control area on the section beach face A, and S4 the SGD and S3 the control area on the section beach face B (Figure 2-1 (C)). SGD seepage has been extensively studied and documented year round at S1 (Leote et al. 2008; Rocha et al. 2009; Ibánhez et al. 2011, 2012). Where SGD seepage occurs, it is visible as water issuing from the sediments and flowing seawards in runnels at low tide. Similar to other studies in this field (e.g. Kotwicki et al. 2013), SGD area S4 was identified by the visual presence of SGD, as was also observed at area S1. For each SGD area, the control area was selected based on proximity to the SGD area (within ~100 m) and complete visual absence of SGD (Figure 2-1 (C)).

SGD and control areas within the same block were located at the same shore height to remove possible differences in ecology associated with shore height. Sampling areas within the same beach face section were located at the same shore height by measuring the distance from the base of the sampling area to the water at low tide and ensuring this distance was similar for the two areas. SGD and control areas were located on the same section of beach face and in close proximity. This is normal in this field of research where distances between SGD and control areas normally range from 10 m to 400 m, depending on local conditions (Kotwicki et al. 2013; Silva et al. 2012; Migné et al. 2011b; Ouisse et al. 2011). Proximity and similarity in hydrodynamic conditions meant that areas on the same beach face section were expected to be more alike in all parameters than areas on different beach face areas, independent of the presence and/or absence of SGD. No biological zonation was visible on the shore surface (please see picture of sampling sites, Appendix A-2).

SGD seepage rates and associated nutrient loads peak in August and November (Leote et al. 2008). To ensure that sampling was conducted following a period of primary producer assimilation of SGD seepage and to avoid the ecological effects of human summer activities in the lagoon, ecological sampling was carried out in early December.

2.3.3. Field sampling – ecological and isotope survey

Fieldwork was conducted during a low spring tide, and over three consecutive days in December 2010. A 5 m^2 area was delineated at each area and subdivided into 1 m^2 units. Random number tables were used to determine which units were sampled. Five samples (cores) were taken at each study area. Core dimensions used in research of this type vary and depend on the complexity and form of the system as well as the information sought. In a previous study of the current system, Aníbal et al. (2007) used cores of diameter 18 cm and depth 2 cm to investigate macroalgae and its related epimacrofauna. It was decided that the subset of the biotic community examined in this study would be that which is associated with surface macroalgae. To accommodate this, the core dimensions employed by Aníbal et al. (2007) were used in the current study. A cut-off bucket with internal diameter 18 cm was pushed into the sediment to 2 cm depth and twisted several times to sever any algae spanning the study and non-study area under the bucket. All above ground material and sediment to 2 cm depth was harvested. Samples were placed in polyethylene bags, stored in a cooler, and returned to the lab within two hours of collection.

2.3.4. Ecological sample processing

Ecological samples collected in the field sampling campaign were processed to test null hypothesis (Ho) (1) and (2). Samples were sieved (0.5 mm) while immersed in a bucket of seawater to remove fine particles. Sieves were immersed to less than their wall height to prevent loss of material from overflow. Samples were sifted through and all living organisms removed and identified to the lowest taxonomic level possible. For algae, the number of species and wet weight biomass of each species was recorded. The number of individuals of each faunal species found per sample was counted to determine species abundance. For faunal biomass, wet weight biomass was determined per species by placing all organisms on tissue to remove adherent water and then weighing the individuals on a microbalance. Where a very large number of individuals were found in a sample, biomass was determined for a representative subsample (10 individuals). Weight per individual was then derived from the average weight of the subsample and multiplied by the total number of individuals present to get a value for the whole sample.

For some organisms, it was not possible to obtain biomass data, i.e. the organism was too small for an accurate determination of mass, or so fragile that part/all of the organism was damaged/lost during prior processing steps, or the organism inhabited the shell of another organism from which it could not be extracted. Where possible, data from other samples and/or areas was used to estimate these results. Missing and estimated data are indicated in Table 2-3.

2.3.5. Isotope sample processing

Organisms found during the field sampling campaign were subject to stable isotope analysis in order to test null hypotheses (Ho) (3) and (4), and tissue %C and %N were also determined. Algae were washed in distilled water to remove sediments and immediately dried at 60°C. Individuals of *Bittium reticulatum* (da Costa, 1778) were held overnight in filtered seawater to depurate gut contents before being transferred to a 60°C oven. All samples were left at 60°C for 48 hours to ensure complete desiccation. Samples were then manually ground to a fine homogenous powder using an agate mortar and pestle. All equipment was trice cleaned with ethanol and allowed to air dry between samples. Ground, desiccated *B. reticulatum* samples were acidified with 1 M HCl, drop by drop until effervescence stopped to remove carbonates which might otherwise alter the C isotopic signature (as per Vizzini and Mazzola, 2003; Carabel et al., 2006; Colombini et al., 2011). After effervescence ceased, samples were immediately transferred to a 60°C oven for 48 hours to re-dry. All ground samples were stored in a desiccator until stable isotope analysis.

2.3.5.1. Isotopic and elemental analysis

Tissue $\delta^{15}N$, $\delta^{13}C$, %C and %N were analysed using a Thermo-Finnigan Delta V Advantage Isotope Ratio Mass Spectrometer (IRMS) online with a Costech Elemental Analyser (EA) at the University of Durham (SIBL - Stable Isotopes in Biogeochemistry Laboratory). Algal material (2.0 - 2.5 mg) and *B. reticulatum* material (2.9 - 4.1 mg) were weighed into tin capsules using a microbalance.

Nitrogen isotopic signatures are reported using δ notation in permil (‰) deviations from the standard, atmospheric nitrogen (N_2), where:

$$\delta^{15}N\ (‰) = [(^{15}N{:}^{14}N_{sample}/^{15}N{:}^{14}N_{atmN2})\ -1] \times 10^3$$

Carbon isotopic signatures are expressed similarly with results reported in permil (‰) deviations from the standard Vienna PeeDee Belminite Limestone (vPDB) where:

$$\delta\ ^{13}C\ (‰) = [(^{13}C{:}^{12}C_{sample}/^{13}C{:}^{12}C_{vPDB})\ -\ 1] \times 10^3$$

Blue nitrile gloves were worn at all stages of material sampling and processing, and all equipment cleaned with ethanol and allowed to air dry to prevent human or cross-sample contamination. The analytical error, derived from the standard deviation of replicate measurements of six different standards, was +/-0.1 ‰ for both $\delta^{13}C$ and $\delta^{15}N$. Elemental concentrations of organism tissue (%C and %N) were calculated using dry weight, and molar C:N ratios determined.

2.3.6. Data analysis

Due to relatively small sample sizes (n = 5 to 10 for ecological analysis, and n = 3 to 13 for isotopic analysis) and demonstrated lack of normality in some cases (Anderson-Darling test, $p < 0.05$), non-parametric statistics were employed for all data analysis. ANOSIM (analysis of similarity, R test statistic, n = 10) multivariate analysis was used to test for an effect of SGD on specific taxa composition/abundance and biomass while incorporating the large number of variables (species) to test null hypotheses (1) and (2). ANOSIM (R test statistic, n = 10) was also used to test for an effect of blocking.

Non-metric multidimensional scaling (nmMDS) ordinations were produced to allow visualisation of the data. nmMDS ordinations are distance based, where similar samples locate close together, i.e. distance is proportional to the degree of dissimilarity. All of the multivariate data contained in any one sample is represented by one point only. This multivariate ordination technique allows the multivariate data contained within each of ten core samples from block beach face A (indicate 'A') and each of the ten core samples from block beach face B (indicated 'B') to be incorporated into one plot. Each of the ten SGD samples and each of the ten control samples are distinguished using symbols, with one symbol indicating 'SGD' and other 'control' samples. A stress value is given for each ordination. The 'stress' indicates how well the ordination represents the underlying data. Stress values < 0.05 indicate an excellent representation with no prospect of misinterpretation and < 0.1 correspond to a good ordination with no real prospect of misleading interpretation (Clarke and Warwick 2001, pp.5–6). nmMDS and ANOSIM were conducted on Bray-Curtis based similarity resemblance matrices constructed from untransformed algae data to test null hypothesis (Ho) (1), and fourth root (√√) transformed macroinvertebrate data to reduce skewness (Clarke and Warwick 2001) to test null hypothesis (Ho) (2), using 'SGD' or 'block' as the factor. All multivariate analyses and computation of diversity indices (species richness *(S),*Simpsons's *(D),* Shannon-Wiener *(H'*)) were carried out using PRIMER v6 (Clarke and Gorley 2006). All diversity indices were computed based on macroinvertebrate species abundance data.

Univariate Wilcoxon rank sum tests (W test statistic) were used to compare small independant samples (individial species abundance data and isotope data) across SGD and control areas to test null hypotheses (Ho) (3) and (4). All univariate statistics were conducted using SigmaPlot Version 12.3 or R (R Development Core Team 2011).

The *nortest* package in R was used for Anderson-Darling tests and all other tests were inherent in R and don't require packages to be loaded. A significance level of 95% ($\alpha = 0.05$) was set as the threshold for all tests. SGD was the primary factor under consideration; however an effect of beach face section (block) was intuitively expected and thus tested for. ANOSIM identified a significant effect of beach face section (block) in all cases. This prohibited inter-beach face section comparisons, restricting univariate comparisons to areas within the same one beach face section only.

2.4. Results

2.4.1. Ecological results

2.4.1.1. Algae – composition and biomass

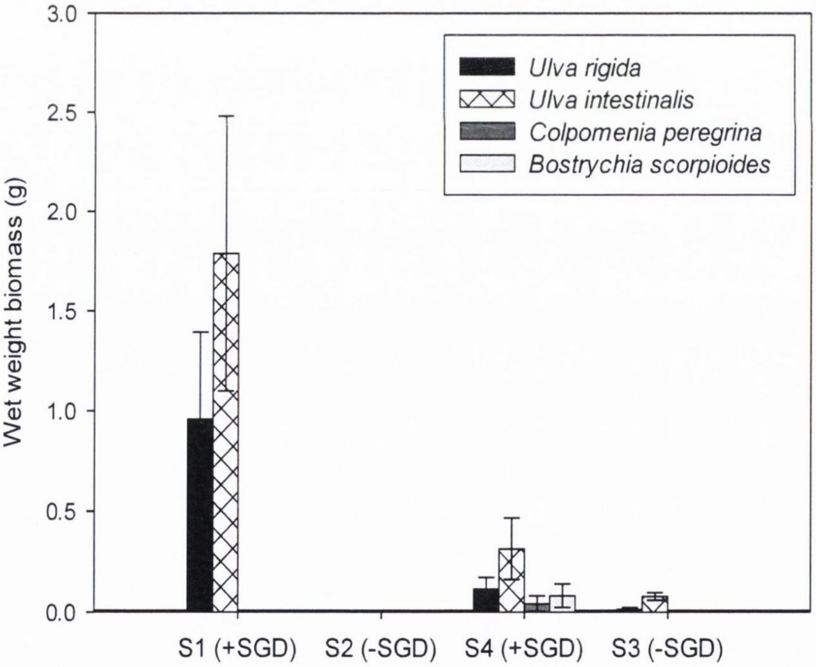


Figure 2-2 Mean (± 1 SE) of algal species wet weight biomass (g) (*n* = 5). S1 (+SGD) and S2 (-SGD) were on beach face section A, and S4 (+SGD) and S3 (-SGD) on beach face section B. No algae were found at S2 (-SGD).

A greater number of algal species, and algal biomass were found at SGD areas (S1 and S4) compared to control areas (S2 and S3). Four algal species were recorded in total. *Ulva intestinalis* (Linnaeus, 1758) was the algae present in greatest biomass on all areas (Figure 2-2). Algae were found attached to shells and debris and formed small tufts comprising algae, small shells, shell particles and sand. Four algal species were recorded at SGD area S4, and two at each SGD area S1 and control area S3. The greatest algal diversity was recorded at SGD area S4 and largest algal biomass at SGD area S1, while no algae were found in samples from control area S2 (Figure 2-2). ANOSIM identified that the algal assemblage differed at SGD compared to control areas (R = 0.46; p = 0.003; n = 10) and there was an effect of the blocking factor 'beach face section' (R = 0.62; p < 0.000; n = 10). Thus null hypothesis (Ho) 1 is rejected as there is a difference in the composition and biomass of the harvested macroalgae community between the SGD and control sites.

2.4.1.2. Macroinvertebrate – structure and abundance

Table 2-1 Mean (± 1SE) abundance of macroinvertebrate species per 0.15m^2 (number of individuals/0.15m^2) (*n* = 5). Entries are tabulated in order of decreasing abundance, and the most abundant species per site are highlighted in bold. SE is not given where one individual was found in one sample (mean = 0.2), where one individual was found in each of two samples per site (mean = 0.4), and so on for one individual in each of three (mean = 0.6) and four samples (mean = 0.08) per site. A mean of 1 indicates that 1 individual was found in each of the five samples and thus the SE is 0. *Species total indicates the total number of species recorded at a given site. **Sample mean is the mean (±1SE) number of species recorded per sample at a given site (*n* = 5).

	Beach face A		Beach face B	
Species	**Site 1 (+SGD)**	**Site 2 (-SGD)**	**Site 4 (+SGD)**	**Site 3 (-SGD)**
Bittium reticulatum	**8.2 (± 3.3)**	**43.8 (± 27.4)**	**163.2 (± 80)**	**6.8 (± 1.2)**
Hydrobia ulvae	**0.6**	**0.6**	**6.4 (± 1.4)**	**8 (± 3.2)**
Cerastoderma edule	**4.25 (± 1.4)**	**1.8 (± 0.6)**		**0.4**
Phascolion strombi	**0.2**	**0.2**	**4.6 (± 2.4)**	
Melita palmata	2 (± 1.1)		0.2	
Paguristes spp.		0.4	1.5 (± 0.6)	
Nassarius reticulatus	1.8 (± 1.1)			
Cyclope spp.			0.6 (± 0.2)	1 (± 0)
Calyptraea chinensis	0.2		1 (± 0)	
Gibbula umbilicalis	0.8		0.8	
Sphaeromatid		0.2	0.8	
Tapes decussatus	0.2	0.4	0.75 (± 0.2)	
Cumacean	0.4	0.2	0.6	
Anguilla anguilla	0.4		0.2	
Cyathura carinata	0.2		0.2	
Amphipholis squamata	1			
Acanthochitona crinitus			0.4	
Spisula subtruncata			0.2	
Ophiura ophiura				0.2
Carcinus maenas	0.2			
Clibanarius erythropus	0.2			
Gibbula varia	0.2			
Owenia fusiformis		0.2		
Turritella communis		0.2		
Venus verrucosa		0.2		
Mesalia brevialis	0.2			
Species total *	17	11	15	5
Sample mean** (± 1SE, *n* = 5)	6 (± 1.3)	4 (± 1.26)	8 (± 0.55)	3 (± 0.55)

In total, 26 macroinvertebrate species were recorded in the study (Table 2-1). A greater mean (number of species/sample) and total number of species were recorded from SGD than respective control areas. The difference was significant for beach face B (W = 0; *p* = 0.01) but not for beach face A (W = 6.5; *p* = 0.24), *n* = 5 in all cases. *B. reticulatum* was the most abundant species at S1, S2 and S4, and the second most abundant species at S3. Other species present in high abundance were *Cerastoderma edule* (Linnaeus, 1758) (at S1 and S2 in particular) and *Hydrobia ulvae* (Pennant, 1777) (at S3 and S4 in particular) (Table 2-1).

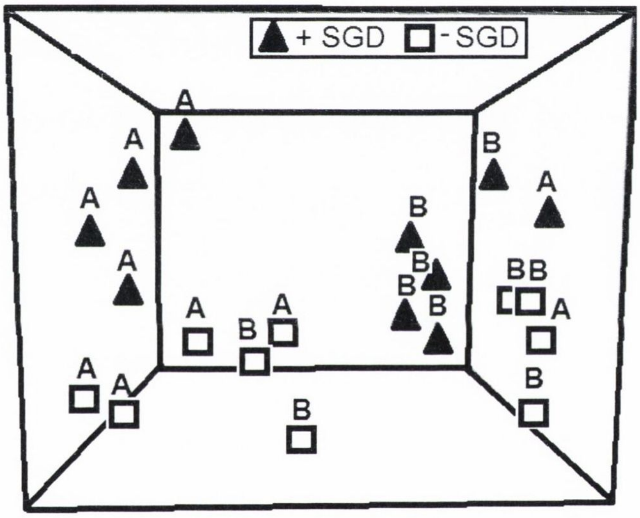


Figure 2-3 3D non-metric multidimensional scaling plots (nmMDS) of underlying Bray-Curtis similarity matrix constructed from fourth root transformed macroinvertebrate abundance data; *n* = 10. '*A*' indicates samples from beach face section A, and '*B*', samples from beach face section B. Stress: 0.09

An increased abundance of individuals of macroinvertebrate species was found in SGD samples. Based on the abundance and composition of macroinvertebrate species, ANOSIM analysis identified a significant difference between SGD and control areas (R = 547; *p* = 0.002, *n* = 10). Thus null hypothesis (Ho) (2) of no difference in structure and composition of the non-sessile macroinvertebrate assemblage between SGD areas and control areas is rejected. ANOSIM analysis also identified a significant effect of blocking (R = 485; *p* < 0.000, *n* = 10). A three dimensional (3D) nmMDS ordination of abundance data shows good separation of samples from SGD and control areas, with relatively low associated stress (0.09) (Figure 2-3). This plot illustrates the relative effect the factor 'block' (along the x - axis) and 'SGD' (y - axis) on the contained systematic variation (Clarke 1993). Samples from block 'beach face section A' (labelled 'A') group together, as do those from block 'beach face section B' (labelled 'B'). Similarly, SGD samples tend to group together, as do control samples (Figure 2-3).

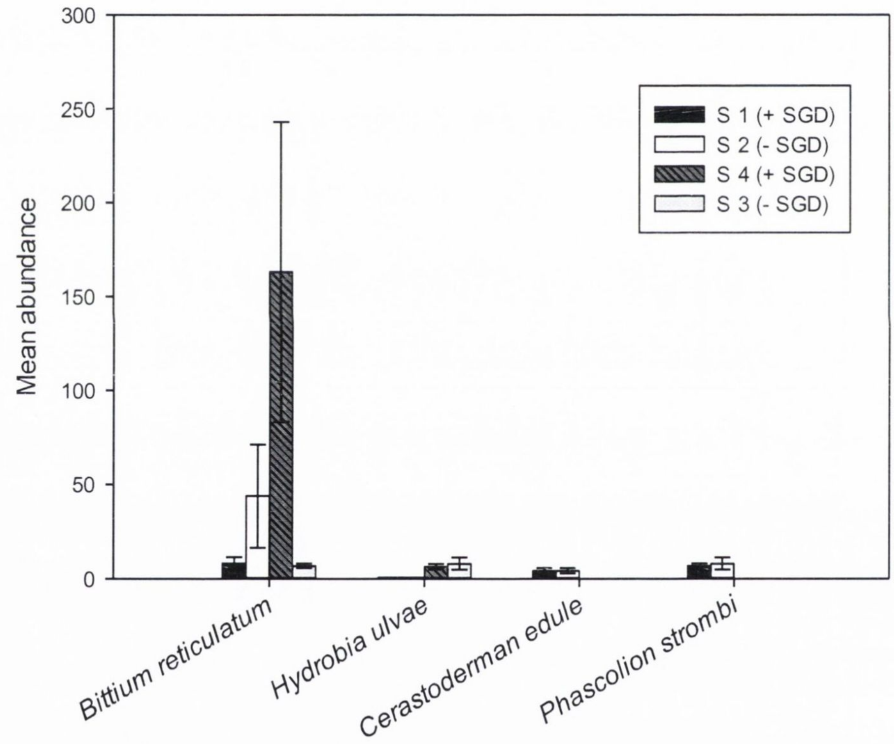


Figure 2-4 Mean (± 1SE) of four most abundant species found across four sites. Data based on table 2-1.

All of the four most abundance species were sampled from both the SGD and control areas, with no apparent patterns in distribution of organisms between SGD and control areas (Figure 2-4). Differences between SGD and control areas were thus due to the presence/absence of rarer species which were recorded from either SGD or control areas only. Five species were found at SGD areas only and at both SGD areas. These were *Melita palmata*¸ *Calyptraea chinensis, Gibbula umbilicalis,* Anguilliformes and *Cyathura carinata*. A number of species were found only at the control area on one beach face, however, no species were found at only the control areas on both beach faces.

Table 2-2 Diversity indices for SGD areas (S1 and S4) and control areas (S2 and S3) on two beach face sections (A and B). All indices are based on the multivariate macroinvertebrate species abundance dataset.

		Diversity Index		
Beach face section	Site	Species richness(S)	Simpson's (D)	Shannon-Wiener (H')
Beach face A	S1 (+ SGD)	19.00	5.84	2.18
	S2 (-SGD)	12.00	2.84	0.51
Beach face B	S4 (+ SGD)	19.00	3.45	0.60
	S3 (- SGD)	7.00	2.08	1.27

There was greater species richness (S) at the SGD than control sites on both beach faces (Table 2-2). Similarly, the value of the Simpson's index was greater at the SGD than control areas on both beach faces. The Shannon-Wiener value was greater at the SGD than control area on beach face A, but lower at the SGD area relative to the control area on beach face B.

2.4.1.3. Macroinvertebrate - diversity and biomass

Table 2-3 Mean macroinvertebrate wet weight biomass (g) per 0.15m^2 (± 1SE expressed as a % of the mean), *n* = 5. SE is not reported where the species was recorded in only one sample. Species present in highest biomass are highlighted in bold.

	Beach face A		Beach face B	
Species	S1 (+ SGD)	S2 (-SGD)	S4 (+SGD)	S3 (-SGD)
Cerastoderma edule	**68 (± 52%)**	**4.2 (± 32%)**		**1.1 (± 62%)**
Bittium reticulatum	**1.4 x10^{-1} (± 44%)**	**4.2x10^{-1} (± 59%)**	**3.2 (± 51.59%)**	**7.4x 10^{-2} (± 33%)**
Tapes decussatus	**6.2x10^{-1}**	**1.4x10^{-1} (± 75%)[a]**	**1.7x10^{-1} (± 53%)**	
Hydrobia ulvae	**7.3x10^{-3} (± 67%)[a]**	**4.3x10^{-3} (± 44%)**	**4.6x10^{-2} (± 18%)**	**7.4x 10^{-2} (± 40%)**
Cumacean	6.0x10^{-4}[a]	3.0x10^{-4}	9.0x10^{-4} (± 40%)[a]	
Cyclope spp.			1.7 (± 43%)	8.4x 10^{-2} (± 45%)
Calyptraea chinensis	8.4x10^{-1}		7.2x10^{-2} (± 50%)	
Gibbula umbilicalis	2.9x10^{-1}		9.7x10^{-2} (±61.5%)[a]	
Anguilliformes	1.3x10^{-1}		6.7x10^{-3}[a]	
Melita palmata	5.3x10^{-3} (± 47%)		5.0x10^{-4}	
Sphaeromatid		1.7x10^{-3}	4.6x10^{-3} (± 43%)[a]	
Paguristes spp.		8.0x10^{-4} (± 61%)[a]	2.4x10^{-3} (± 49%)[a]	
Carcinus maenas	5x10^{-2}			
Clibanarius erythropus	1.6x10^{-3}			
Acanthochitona crinitus			6.7x10^{-3} (±92%)	
Amphipholis squamata	1.9x10^{-3} (± 77%)[a]			
Gibbula varia	3.3x10^{-2}			
Nassarius reticulatus	2.6 (± 66%)			
Spisula subtruncata			4.6x10^{-2}	
Ophiura ophiura				1.1x 10^{-1}
Turritella communis		1.4x10^{-1}		
Phascolion strombi	[b]	[b]	[b]	
Cyathura carinata	[b]		[b]	
Mesalia brevialis	[b]			
Owenia fusiformis		[b]		
Venus verrucosa		[b]		

[a]Some data missing but representative values obtained from averages of the same species in other samples. [b]Data missing.

Generally, the biomasses of individual macroinvertebrate species were larger at SGD than respective control areas (Table 2-3). There were however some exceptions: *Paguriste* sp., a member of the Sphaeromatidae and *Turritella communis* (Risso, 1826) found at S2, and *C. edule* and *Ophuira ophiura* (Linnaeus, 1758) found at S3, while *Cyclope* sp. was present in greater biomass at S3 than S4 (Table 2-3). ANOSIM identified a significant effect for the blocking factor 'beach face section' (R = 0.393; p = 0.002, n = 10), however the result for SGD was just above the limit set for statistical significance (R = 0.138; p = 0.06, n = 10). Therefore, null hypothesis (Ho) (2) cannot be rejected on the basis of macroinvertebrate diversity and biomass. There is no statistical difference in the macroinvertebrate diversity and biomass between SGD and control sites. To permit inclusion in analysis and ordinations, missing samples were assigned a weight of 0.0001g. This negligible weight has little impact in terms of biomass, however, the presence of the species is relevant as both the ANOSIM test and nmMDS ordination are based on assessment of biomass and assemblage composition.

2.4.2. Isotopic ($\delta^{15}N$ and $\delta^{13}C$) results

To enable direct comparisons, only species which met all three of the following criteria were used for isotopic analysis; (1) sampled in sufficient biomass to allow minimum of two replicates per area, (2) sampled from at least three of the four areas, and (3) species, though possibly different, belonging to the same trophic level. Only three species met these requirements, the ephemeral green algae *Ulva rigida* (Agardh, 1823) and *U. intestinalis* (Linnaeus, 1753) (as these belong to the same genus and functional guild, algae from the same sample were pooled for analysis), and the infaunal detritivore *B. reticulatum*. Ephemeral green macroalgae were found at S1, S4 and S3. *B. reticulatum* were sampled at all areas.

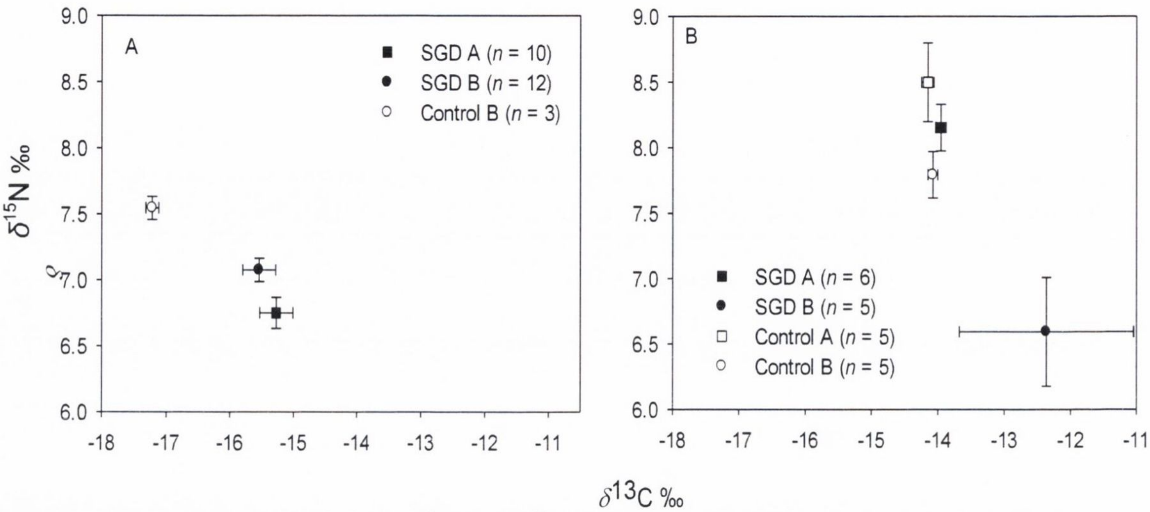


Figure 2-5 Scatter plot of mean δ^{15}N and δ^{13}C (± 1 SE) of (A) *Ulva* spp. at SGD areas S1 and S4, and control S3, and (B) *B. reticulatum* at SGD areas S1 and S4, and control S2 and S3.

Algal δ^{15}N and δ^{13}C values differed between SGD area S4 and control area S3 (Figure 2-4 (A)). S4 algae had lower δ^{15}N (W = 3.29; p = 0.01) and higher δ^{13}C values (W = –5.2; p < 0.000) than S3 algae. Algae at SGD area S1 (n = 10) were also depleted in ^{15}N and enriched in ^{13}C compared to the algae at control area S3, though they were not compared statistically due to differences between beach face sections. Thus null hypothesis (Ho) (3) and (4) can both be rejected for the macroalgal component of the food web, as the nitrogen and carbon isotopic signatures of SGD macroalgae differed statistically from that of control food web macroalgae.

Depletion in ^{15}N and enrichment in ^{13}C at SGD areas was also visible in *B. reticulatum* tissue, though differences were less pronounced (Figure 2-4 (B)). Only for δ^{15}N at S4 and S3 on beach face A was the difference statistically significant (Wilcoxon rank sum; W = 2.67; p = 0.04) with the means of the two areas differing by 1.2 ‰, and the 95 %

C.I. for the difference between the two means ranging from 0.07 ‰ to 2.32 ‰. Thus null hypothesis (Ho) (3) can only tentatively be rejected for macroinvertebrates. Null hypothesis (Ho) (4) cannot be rejected for macroinvertebrates as the carbon isotopic signature of macroinvertebrate components of the food web did not statistically differ between the SGD and control food web.

Following acidification, *B. reticulatum* sample material became hygroscopic due to the presence of the desiccant $CaCl_2$. This may have led to some sorption of atmospheric moisture by the samples post-drying. Sorption of varying amounts of water may have resulted in minor inaccuracies in the weights of the sample weighed into the tin capsules prior to isotopic analysis. This could have resulted in a higher degree of variation in the results than expected from a dry sample. This might have inflated the standard deviation of the mean isotopic values associated with these samples.

The expected isotopic enrichment per trophic level (i.e. trophic fractionation) is 0 to 1 ‰ for carbon (DeNiro and Epstein 1978; Peterson and Fry 1987), and 2.5 to 3.4 ‰ for nitrogen (Zanden and Rasmussen 2001; Vanderklift and Ponsard 2003; DeNiro and Epstein 1981). In all cases the difference in isotopic signature between macroalgae and *B. reticulatum* sampled from the same one area was greater than 1 ‰ for C, at ~1.3 ‰ for S1 (+ SGD), ~3 ‰ for S2 (- SGD) and ~4 ‰ for S4 (+ SGD). The difference in macroalgae and *B. reticulatum* nitrogen isotopic signature was ~1.5 ‰ for S1 (+ SGD), 0.5 ‰ for S2 (- SGD) and 0 ‰ for S4 (+ SGD). The non-overlap between the observed differences in isotopic signatures of macroalgae and *B. reticulatum* for both carbon and nitrogen and the expected trophic enrichment factors for carbon and nitrogen indicates that macroalgae contributed either minimally or not at all to the diet of *B. reticulatum*. Though the two trophic levels sampled belong to the same food web they are largely independent of each other in terms of energy and nutrient trophic transfer.

2.4.3. Elemental results

2.4.3.1 Algae

Table 2-4 Elemental composition and C:N molar ratio (mean and 1SE) of algal tissue collected at the SGD area on beach face A (S1), and the SGD and control areas on beach face B (S4 and S3 respectively).

	S1		S4		S3	
	Mean	**SE**	**Mean**	**SE**	**Mean**	**SE**
n	10		13		3	
%C	14.69	0.94	15.69	0.18	12.48	0.79
%N	1.73	0.13	1.73	0.11	1.33	0.11
C:N	9.93	0.19	10.57	0.11	11.02	0.29

Comparison of the SGD area and control area on beach face B (i.e. S4 and S3 respectively) using Wilcoxon rank sum tests identified no difference in algal tissue N content ($W = 8$; $p = 0.14$), C content ($W = 11$; $p = 0.28$), or C:N ratio ($W = 8$; $p = 0.14$) between sites. Algae at SGD S1 on beach face A were not compared to the algae at control S3 on beach face B as they were sampled from different beach face sections.

2.4.3.2 Macroinvertebrate

Table 2-5 Elemental composition and C:N molar ratio (mean and 1SE) of *Bittium reticulatum* tissue collected at the SGD area (S1) and control area (S2) on beach face A. * indicates significant at $\alpha = 0.05$.

	S1		S2			
	Mean	**SE**	**Mean**	**SE**	***W***	***p***
n	6		5			
%C	2.47	0.08	2.66	0.11	5	0.08
%N	0.60	0.02	0.65	0.03	7.00	0.18
C:N	4.77	0.04	4.80	0.03	12.00	0.66

Table 2-6 Elemental composition and C:N molar ratio (mean and 1SE) of *Bittium reticulatum* tissue collected at the SGD area (S4) and control area (S3) on beach face B. * indicates significant at $\alpha = 0.05$.

	S4		S3			
	Mean	**SE**	**Mean**	**SE**	***W***	***p***
%C	2.59	0.32	2.24	0.19	5	0.15
%N	0.50	0.04	0.56	0.03	9	0.56
C:N	6.18	0.76	4.66	0.13	1	0.02*
n	5		5			

Only for the C:N ratio of *Bittium reticulatum* at beach face B did the samples differ statistically between the SGD and control sites (Wilcoxon rank sum).

2.5. Discussion

The presence of SGD was associated with altered structure and composition of a section of the benthic intertidal food web at a sandy beach face. Furthermore, Algal diversity and biomass were elevated at SGD compared to control areas.

The study herein is subject to a number of caveats relating largely to (1) the restricted subset of the food web sampled and (2) the choice of sampling sites in the absence of water chemistry and nitrogen data. A relatively small subset of the benthic food web, and for some species a restricted number of individuals, were sampled. This hampered ecological and isotopic statistical comparisons. The reduced assemblage may have to some extent been an artifact of the sampling season and methodology. The current study was conducted in one season only, i.e. winter. This was in order to ensure that sampling took place when SGD seepage and nutrient loading was at its highest (summer and winter (Leote et al. 2008)) while avoiding the ecological effects of human summer activities in the lagoon. It has been observed that periods where adult macrophyte coverage are absent are very short in the Ria Formosa (Schories et al. 2000). Also, it has been noted that the lagoon does not display the expected seasonality of macroalgae cover and development (Schories et al. 2000). Despite these observations, there may be an influence of seasonality on the benthic food web, which may have resulted in more limited food web in winter (Akin and Winemiller 2006). Future research should endeavor to sample with greater temporal replication, ideally in two to three seasons, over two years.

The cores used in the current study had diameter 18 cm and depth 2 cm, similar to those used by Aníbal et al. (2007) in previous research of the benthic intertidal food web in the lagoon. Core dimensions used in research of this type vary and depend on the complexity and form of the system as well as the information sought. For example, Ouisse et al. (2011) in their study of SGD sampled three quadrats of 0.1 m^2 and 10 cm depth for macrofauna <2 mm, and three sediment cores of diameter 2.9 cm^2 and 2 cm depth for meiofauna. In other subtidal SGD research, Encarnação et. al. (2013) took cores of 3.5 cm diameter and 15 cm depth to investigate meiofauna. In light of the relatively limited subset of the food web assemblage, and the restricted number of individuals sampled in the current study, future research might employ cores of smaller diameter and greater depth in order to sample a greater subset of the benthic assemblage. Also, species and individuals found deeper in the sediment may be of larger size and biomass. Thus, sampling to a greater depth may alter the biomass results, which were found to not significantly differ between SGD and control areas in the current study.

No water chemistry data or samples for nutrient analysis were taken at the SGD areas. SGD and control areas were identified based on the visual absence or presence of water issuing from the sediments and flowing toward the sea in runnels. The study relies on algal and macroinvertebrate isotope data to infer that the SGD contained a freshwater component which was nitrogen enriched. The nitrogen enrichment of SGD at S1 is corroborated by porewater nitrogen analysis at this area which ran concurrent to the sampling campaign documented herein (NitroLINKS 2007-2011) (Table 2-7). This water sampling campaign measured porewater nitrate, nitrite and ammonia concentrations during a 10.5 hour time period along a 16 m transect at S1 in December 2010 (NitroLINKS 2007-2011). Piezometers were used to sample porewater at depths of ~0.5 to 3.5 meters. Salinity measurements were also taken, however there was an error with the salinity probe. The exact salinities are unknown, but the salinity varied by approximately 4.5 units among samples. This indicates at least some fraction of freshwater, however the exact proportion is unknown as the actual value of the highest salinity samples is unknown, i.e. the highest salinity measured may have been 35 psu, or, for example 20 psu with the 4.5 psu decrease relative to the initial salinity. From this data, the SGD was enriched in nitrogen, mostly in the form of nitrate, and at times of reduced salinity at the time of sampling. Similarly, Leote et al. (2008) found that the SGD at area S1 in December was of variable and sometimes reduced salinity (range 30.9 to 37.7 psu), and contained nitrate at concentrations ranging from 1.3 to 49.9 µM and no detectable ammonium. Similar water chemistry was assumed for the SGD area on beach face B in the current study. Future research should however include a full battery of water chemistry (salinity, temperature, pH), nutrient (nitrate, nitrate, ammonia, phosphate) and isotope ($\delta^{15}N_{NO3-}$ and $\delta^{18}O_{NO3-}$) analysis in order to fully characterise the SGD.

Table 2-7 Porewater nitrate (NO3-), nitrite (NO2-) and ammonium (NH4+) data from December 16th 2010. *n* = 50 in all cases. Only ten of the 50 samples contained nitrite at a detectable concentration and only five of the 50 samples contained ammonium at a detectable concentration.

Species	Concentration (µM)	SE	Range(µM)
NO_3^-	54	18	3 - 265
NO_2^-	0.031	0.004	0 - 0.208
NH_4^+	0.084	0.041	0 - 3.26

Primary producers comprise the lowest trophic level and hence determine the composition and structure of the higher trophic tiers. Sandy beaches generally have little *in-situ* primary production (McLachlan and Brown 2010) with primary resources derived largely from phytoplankton, macrophytes detritus, carrion and stranded algae (Bergamino et al. 2011). In this study however, macroalgal surface assemblages were present at SGD areas. Links between primary production and SGD have previously been documented. For example, Johannes (1980) reviewed the tentative preliminary findings of a small number of experiments which indicated the potential significance of SGD in determining the composition and distribution of seagrass, mangrove stands and rooted salt marsh plants. Kamermans et al. (2002) found an inverse relationship between subtidal groundwater seepage and seagrass species diversity and none between SGD and seagrass abundance or biomass in East African lagoons. Migné et al. (2011a), on the other hand, found an increase in primary production at an intertidal SGD site in Roscof bay, France, congruent with the results of our study. SGD may influence the composition of the primary producer trophic level. Recruitment and proliferation of some guilds/species, such as sea grasses, may be inhibited by SGD (Kamermans et al. 2002), possibly due to reduced light visibility associated with SGD-enhanced eutrophication. On the contrary, SGD may enable and perhaps promote settlement of other species, such as the opportunistic primary producer guilds/species as observed in the current study and elsewhere (Valiela et al. 1990; Ouisse et al. 2011).

Species from the genus *Ulva* comprised the largest algal biomass present at all three sites (both SGD sites and one control site). Valiela et al. (1990) also found dominance and proliferation of *Ulva* spp. associated with SGD seepage in early investigations at Waquoit Bay, Cape Cod. These results are relevant for SGD seepage areas in general, and for shallow lagoons and embayments in particular. The impact of SGD, both chemical and ecological, may be heightened in smaller bodies of water such as embayments and lagoons due to their limited volume and restricted water exchange with the open ocean. Release from nutrient-limitation in these shallow systems can result in benthic primary production which is significant in terms of whole ecosystem production and functioning in large tidal flat ecosystems (Waska and Kim 2010a), where it can constitute more than 60% of primary production (Lee and Olsen 1985). Salinity is considered a major factor controlling the occurrence and proliferation of *Ulva* spp. (Martins et al. 2001). The salinity of SGD in the lagoon, where occurring, has previously been recorded to range from 17 to 36 psu (Leote et al. 2008). *Ulva* spp. proliferate at salinities between 20 – 30 psu, particularly in combination with elevated nitrogen concentrations (Martins et al. 2001). Under such conditions the negative growth effect imposed by reduced salinity is often overcome or offset by increased nitrogen availability (Kamer and Fong 2001b), affording these species a competitive advantage over their less freshwater-tolerant marine counterparts. The optimum salinity range for *U. intestinalis* growth is 17 – 22 psu, with very low growth rates at salinities of < 5 and > 25 psu (Martins et al. 1999). The salinity optima for *Ulva* spp. overlap the range of that previously reported for SGD entering the lagoon (Leote et al. 2008). SGD is often brackish in nature and nitrogen enriched (e.g. Carruthers et al. 2005; Charette and Buesseler 2004; Hays and Ullman 2007; Leote et al. 2008; Taniguchi et al. 2002; I. Valiela et al. 1990), thus we might expect *Ulva* spp. and other algal species with similar life histories to frequently occur in areas of SGD, as was the case in the current study. *Ulva* spp. is not however considered a key species for identifying SGD. *Ulva* spp. do occur in fully marine environments (Dickson et al. 1982), however it is competitively superior relative to less freshwater tolerant species in areas influenced by freshwater (particularly combined with high nitrate concentrations (Choi et al. 2010). Thus, while *Ulva* spp. cannot necessarily be considered an indicator of freshwater SGD input, it is reasonable to expect increased proliferation of these species in areas of freshwater SGD input, relative to control areas where they may also occur.

As the study herein was observational rather than manipulative, the strict conclusion is not that SGD is necessarily casual to the community change, though it is a very plausible mechanistic explanation (Clarke 1993). Rather, the conclusion is that there is statistical evidence of an association between the presence of SGD and the differences in the ecological community (Clarke 1993). The exact mechanisms of the ecological change have not yet been elucidated. The effect of SGD on the algal community for example, (i.e. greater diversity and biomass at SGD relative to control areas) may be due to reduced predation at SGD sites due to conditions unfavorable to consumers (i.e. top down control). Alternatively, the observed pattern may be due to increased growth of algae due to conditions which are favorable for algae growth (e.g. increased nutrient supply) at the SGD relative to control sites (i.e. bottom up control). The observed pattern in macroalgal diversity and biomass is likely to be determined by a dynamic balance between the effects of top down and bottom up forces.

Macroinvertebrate biomass, species number, and abundance of individuals of any single species were generally higher at SGD than control areas. The species diversity indices corroborate the results of the multivariate analysis. Greater species richness (*S*) was recorded at the SGD areas on both beach face sections. Species richness was 36 % greater at the SGD relative to the control area on beach face section A, and 63 % greater at the SGD than the control area on beach face section B. The value of the Simpsons index (*D*) was also greater at the SGD relative to control sites on both beach face sections. The Simpson's index (*D*) was 50% greater at the SGD relative to the control area on beach face section A, and 39 % higher at the SGD relative to the control area on beach face section B. The value of the Shannon-Wiener (*H'*) was greater at the SGD relative to the control area on beach face section A (by 164%), however it was 192 % lower at the SGD relative to the control area on beach face section B. The Shannon-Wiener index incorporates the degree of evenness in species abundances while emphasizing the species richness (Magurran 2004, p.114). Simpson's index (*D*) on the other hand is weighted by the abundances of the commonest species and emphasizes the dominance as opposed to richness component of diversity (Magurran 2004). The contrasting result for the Shannon-Wiener index at beach face section B relative to the other indices is likely due to the very high abundance of *Bittium reticulatum* at the SGD area relative to the control area, associated with differences in how the indices are weighted. The abundance of *B. reticulatum* at SGD S4 is the highest abundance of any species found in the sampling campaign. The abundance of *B. reticulatum* at SGD S4 is over 3.5 times that of the next highest abundance species sampled in the campaign (*B. reticulatum* at control area S3 on beach face section A). The very high abundance of this species has a strong effect on the Shannon-Wiener index which incorporates the degree of evenness. SGD was associated with increased macroinvertebrate species richness, and greater Simpson's index indicating intermediate frequency of disturbance. The Shannon-Wiener index for beach face B however might suggest a low frequency of disturbance at this site and associated increased dominance of one single species. If SGD is thought of as a form of disturbance, the results suggest that this disturbance occurs at intermediate to possibly low frequency.

Miller and Ullman (2004) also found that SGD altered the community composition on sand flats in the USA, where SGD was associated with dense populations of *Marenzelleria viridis*, a Polychaete worm normally associated with fresh oligohaline conditions. Similarly, Zipperle and Reise (2005) found a significant influence of SGD on community structure when they investigated the relationship between SGD and the distribution and density of three Polychaete worms (*Arenicola marina*, *Neries* *diversicolor* and *Nereis virens*) on intertidal sand flats; *A. marina* was completely absent while there was a 12 x increase in the abundance of the other two Polychaete species at seepage sites. Similar results of the effect of SGD on the abundance Polychaete and Oligochaete worms have been found elsewhere (Silva et al. 2012). In contrast to the current study where SGD was associated with an increased number of species (species richness), a recent intertidal study in Roscof Aber Bay, France, found the seepage site associated with lower species diversity in term of species richness with 6 ± 1 (mean ± SE) species found at the SGD site relative to 11 ± 3 (mean ± SE) at the control site (Ouisse et al. 2011). In a second part of the same study Migné et al. (2011) found no effect of SGD on the total macroinvertebrate biomass. These authors did find however that the biomass distribution among trophic levels differed between the SGD and control sites with SGD associated with a trophic shift from a grazer dominated system (~65% of biomass) to one where grazers and omnivores shared dominance (~45% each) (Ouisse et al. 2011). In the current study, all areas were detritivore dominated with no marked shifts in trophic dominance between SGD and relative control areas. Thus, similar to previous studies in the field, the current research documents a significant effect of SGD on macroinvertebrate community composition and structure.

The salinity documented by Leote et al. (2008) at Site 1, 30.9 to 37 psu, falls within the highest marine salinity bands of both the Venice system (30 – 40 psu) (Venice System 1959) and the system described by Bulger et al. (1993) (16-27 psu and >/= 24 psu). No species were found jointly at the control sites but absent from the SGD sites. Thus, the presence of SGD did not exclude from the SGD sites any marine species which were found at the control sites. The difference between SGD and control sites was strongly influenced by the occurrence of five species at the SGD site only on both beach faces

(*Melita palmata*¸ *Calyptraea chinensis, Gibbula umbilicalis, Anguilla anguilla and Cyathura carinata*). All of these species were relatively rare (mean abundance; 0.2 to 2) and four of these species are characteristically estuarine/freshwater tolerant species. *M. palmata* is found in lagoons, estuaries and brackish environments in the Mediterranean Sea (Lincoln 1979). It tolerates a very wide range of salinity ranges and is thus usually found in lagoons or near river mouths where there is a high influence of freshwater (Karaman 1982; Sconfietti 1989). *G. umbilicalis* is tolerant of emersion and typical of brackish water (Hayward et al. 1996, p.184). *A. anguilla* is tolerant of reduced salinities and common in rivers, estuaries and inshore coastal waters (Hayward et al. 1996, p.312). *C. carinata* is an estuarine species found at salinities as low as 13.5 psu (Marques et al. 1994). Previous work on *C. carinata* has found that abundance of this isopod species is positively correlated with salinity within the range 13.5 to 21 psu and also nitrate concentration (Marques et al. 1994). The salinity imposed by SGD did not exclude marine species, however it allowed for the colonisation of freshwater tolerant/marine species at the SGD sites with four characteristically estuarine/freshwater tolerant species recorded at both of the SGD sites but absent from both of the control sites. The occurrence of these species which are normally found in areas of reduced salinity in the otherwise fully marine lagoon may be used as a potential indicator of the presence of SGD (in the absence of other surface freshwater inputs) in this and other similar systems.

Three main benthic intertidal habitat types exist in the Ria Formosa lagoon based on the dominant production processes; (1) sand flats, (2) mudflats, and (3) *Zostera* (seagrass) beds (Sprung, 1994; Almeida et al., 2008). The faunal composition reflects the quantity and quality of primary production available in each habitat. Sprung (1994) found that of the three habitats, sand flats had the highest species number and greatest species diversity. Sprung (1994) postulated that the high numbers of macroinvertebrates were maintained by macrophyte detritus, however, the current study found a significant *in-situ* algal assemblage at seepage (SGD) areas. It is possible that the sites sampled in 1994 were sites of SGD, and had sand flats devoid of SGD been investigated they might have had lower species numbers and diversity which might have altered the rank of this habitat relative to the other habitat types. In 2008, a similar investigation found contrasting results when monthly faunal density, biomass and species diversity were quantified in the same three habitat types (Almeida et al. 2008). Importantly, this study only considered sand flat sites devoid of surface assemblages/above ground structures. These sites may have been, or are at least comparable to, control sites in the current study. Contrary to the results of Sprung (1994), Almeida et al. (2008) found sand flats to have the lowest macroinvertebrate density, diversity and biomass, while the highest values for all of these parameters were associated with the seagrass habitat. *B. reticulatum* was the most abundant macroinvertebrate species at three sites (S1, S4 and S2) in the current study and for the sand flat habitat in 1994 (Sprung 1994). Almeida et al. (2008) found that the main species on the sand flats devoid of surface cover was the snail *Hydrobia ulvae* (as was the case for control S3 on beach face B in the current study).

H. ulvae was equally abundant at both the SGD and control area on beach face section A, but more abundant at the control area (salinity ~36 psu when immersed) than the SGD area (salinity expected to range from 17 to 36 psu (Leote et al. 2008)) on beach face section B. Previous research has investigated the influence of salinity in determining the distribution and success of *Hydrobia ulvae*. Fenchel (1975) studied the distribution patterns of three Hydrobiidae species (*H. ulvae*, *H. ventrosa* and *H. neglecta*) in a complex, estuarine environment and found that different species of this snail show habitat selection with respect to salinity. Fenchel (1975) found that *H. ulvae* preferred the highest and *H. ventrosa* the lowest salinities, while *H. neglecta* had an intermediate position. The salinity ranges at which these species are generally found in the field are 6-20 psu for *H. ventrosa*, 10-24 psu for *H. neglecta*, and 10-33 psu for *H. ulvae* (Muus 1976). *H. ulvae*'s optimum salinity is 30 psu, though *H. ulvae* is found at salinities as low as 5 psu in the Baltic (Hylleberg 1975) and grew at salinities from 3 - 15 psu during cage experiments in an estuary in the Chupa Bay region of Russia (Gorbushin 1996). Low salinity negatively influences *H. ulvae* larvae, with decreased larval survival and activity at low (12 and 18 psu) relative to higher (28 psu) salinities (Grudemo and André 2001). All three species examined by Fenchel (1975) (*H. ulvae, H. neglecta* and *H. ventrosa*) had wide and overlapping salinity tolerance ranges, although in most areas only one of the species occurred. Though *H. ulva* dominated other Hydrobiidea species at relatively high salinities within an estuarine environment (Fenchel 1976), the boundaries between two species in salinity gradients occurred at different salinities in different areas (Fenchel 1975). These overlapping salinity tolerance ranges of the species lead Fenchel (1975) to conclude that salinity alone does not determine the distribution of these species in the field. Fenchel (1976) suggested that the distribution of these species is a function of habitat selection, interspecific competition, dispersal rates, colonisation and extinctions. Fenchel (1976) demonstrated that interspecific competition between Hydrobiidea species of the same size range is a significant as intraspecific competition. Grudemo and Andre (2001) more recently demonstrated that salinity does not influence *H. ulvae* interspecific competition within the salinity band 15-30 psu. Within this salinity band, competition for resources between Hydrobiidea species as this salinity regime favours *H. ulvae* relative to the other Hydrobiidea species. At salinities either above or below this band, interspecific competition may be of greater importance.

Newell (1964) also studied the behavioral response of *H. ulvae* to salinity and the role of salinity in determining the distribution of this species. Newell (1964) found that salinity played a large part in determining the vertical distribution of *H. ulvae* on the intertidal shore along the Crouch river estuary, UK. *H. ulvae* occurred higher in the intertidal zone in the upper relative to the lower reaches of the Crouch estuary. Its distribution was restricted further to a only narrow band in the upper reaches as exposure to air and dessication imposed a stress in the higher levels of the intertidal zone. Newell (1964) found that the control over *H. ulvae* distribution by salinity was due to physiological responses to low salinity which manifested as changes in the activity of the snails in general, and particularly in their floating response. Generally reduced activity at reduced salinity reduces the tendency to float and the number of animals which float, and floating ceases completely at salinity lower 2.1 psu (Newell 1964). While studying the spatial variation in the benthic macroinvertebrate community found in intertidal seagrass from the fresh to marine section and estuary, Barnes (2012) similarly described a '*Hydrobia* zone' referring to the upper intertidal region of the upstream section of the surveyed area which was dominated by *Hydrobia knysnaensis*, a close relative of *H. ulvae*. Thus, while *H. ulvae* can occur at low salinities, it prefers higher salinities within estuarine environments. This data corresponds to the distribution of *H. ulvae* reported in the current study. Barnes and Greenwood (1978) described the influence of sediment type in determining the distribution of *Hydrobia ulvae*, finding a marked preference for muddy sediments, though later work by Barnes (1979) found that *Hydrobia* populations were not uniform in respect of their behavioural responses to sediment type making inferences about their distribution relative to sediment type more difficult. The sediment size found at the SGD and control area may have differed due to mobilisation of small grain particles (mud and silt) by the issuing

SGD. Thus, the differences in occurrence of *H. ulvae* between the SGD and control site may be due in part to salinity and sediment differences.

B. reticulatum has elsewhere been recorded in areas of salinity 12 to 36.9 psu (Peacock 1993; Albayrak et al. 2006). While *B. reticulatum* is found at fully marine salinities (Albayrak et al. 2006), Peacock (1993) found during a comprehensive review of invertebrate salinity tolerances that *B. reticulatum* normally occurs at salinity ~25 psu. At reduced salinities, *B. reticulatum* may have a competitive advantage as it experiences less stress relative to organisms which require high salinities. Here 'stress' is the reduction in the performance or fitness of an organism due to exposure to environmental conditions (Menge and Sutherland 1987), where salinity imposes a physiological stress (Sanford 2002). The abundance of *B. reticulatum* at the SGD area on beach face section B was ~25 fold greater than that at the control area. However, the abundance of *B. reticulatum* at the SGD area on beach face A was five times lower that that found at the associated control area. Biotic factors such as also influence the occurrence and proliferation of organisms. The distribution of *B. reticulatum* is may also be influenced by biotic/biological factors (e.g. predation and interspecific competition), and other stress-causing abiotic factors (such as wave action). These can lead to variability in abundances within similar environments at relatively small spatial scales (Albayrak et al. 2006). While salinity may explain in part the distribution of this species at some sites within the current study, it's likely that its distribution is also heavily influence by other factors. With reference to the results of the current study and the documented salinity preferences of *H. ulvae* and *B. reticulatum*, the occurrence of small scale intertidal SGD events in the Ria Formosa might explain the contrasting results obtained by Sprung (1994) and Almeida et al. (2008), as well as potentially altering the conclusions of other ecological studies in the lagoon (Schories et al. 2000).

Species abundances and individual biomasses can be seasonally variable. Some invertebrates show increases in individual organism biomass between late winter and early summer, declining during the remainder of the year associated with somatic changes (rather than gemetic release) (Beukema et al. 2014). Dekker and Beukema (2007) found that for the bivalve *Macoma balthica,* organisms gained weight in spring and early summer, but lost up to 70% of this during the subsequent year. Weight loss was inversely correlated with organism age and with water temperature in winter. Weight gain is associated with increased food (primary producer) availability and vice versa for weight loss (primary producer) (Beukema et al. 2014). As well as biotic factors such primary production or food density (Beukema 1976), physical factors which exert a stress may to some extent control species occurrences and biomass. The counteracting effects of the various biological processes and abiotic sources of stress can result in a high degree of variability or stochasticity in organism occurrence, abundance and biomass. These mechanisms may be attenuated by freshwater SGD, which may contain elevated nutrient concentrations and, in the summer months at least, may also be of reduced temperature relative to the marine environment (Wilson and Rocha 2012). Increased nutrient concentrations may promote primary producer growth, potentially exerting a bottom up control over organism occurrence, abundance and biomass at SGD areas. However, the reduced temperature associated with SGD may act as a form of stress which negatively effects these parameters by reducing metabolic processes, which are normally positively correlated with temperature. Thus, the counteracting biological and physical factors associated with SGD may condition the variability or stochasticity in the occurrence, abundance and biomass of organisms both relative to control areas and between SGD sites within the one system. This may for example explain the contrasting results found for the abundance of the species *B. reticulatum* at the SGD and control site between beach face section A and beach face section B.

The causal factors of ecological change associated with SGD have not yet been investigated for any system. As previously discussed, a large number of physicochemical properties vary in SGD relative to the receiving marine environment. Also, SGD may alter the physical environment at the seepage site, e.g. temperature, granulometrics etc. Further, in the case of intertidal SGD at least, the presence of SGD may alter (i.e. reduce) the desiccation stress felt by organisms at the site of discharge upon tidal retreat. Thus, a very large number of causal agents of ecological change may be altered by the presence of SGD. Given the infancy of this field of research, with less than ten published articles investigating the ecological alterations associated with intertidal SGD (Migné et al. 2011a; Dale and Miller 2008; Dale and Miller 2007; Ouisse et al. 2011; Zipperle and Reise 2005), the first objective is to determine if SGD is associated with ecological alterations. Following from this, research should focus on determining the form and magnitude of these alterations. Specifically, this should aim to determine if the alterations are consistent and directional, possibly allowing them to be predicted and tested. Once these questions have been addressed, the next step in the study of the ecology associated with SGD is field and particularly laboratory investigations to determine how the suite of causal agents of change which vary due to SGD individually and synergistically condition the form of ecological alterations associated with SGD.

Estuarine and marine macroalgae range in carbon isotopic signature from - 8 to - 27 ‰ (Michener and Kaufman 2007, p.256; Rogers 2003; Filgueira and Castro 2010; Cornelisen et al. 2007). The literature reported nitrogen isotopic values for *Ulva* spp. sampled from relatively unpolluted areas range from ~6 to 9 ‰ (Rogers 2003; Macko et al. 1982; Monteiro et al. 1997; Cornelisen et al. 2007; Filgueira and Castro 2010). The literature reported values for carbon and nitrogen isotopic signature of *B. reticulatum* vary widely, from - 9.5 to - 19 ‰ for δ^{13}C, and 3.3 to 10.5 ‰ for δ^{15}N (Pinnegar and Polunin 2000; Gacia et al. 2009; Lepoint et al. 2000; Ogden et al. 2006). Thus, the isotopic signatures found in the current study are within the expected literature ranges for both macroalgae and *B. reticulatum*. Isotopic signatures are variable and highly conditioned by the local system conditions and parameters of the carbon and nitrogen sources. Although the literature report ranges encompass the values of all isotopic signatures found at all sites in the current study, SGD and control areas differ in their isotopic signatures.

Both algae (*Ulva* spp.) and *B. reticulatum* at SGD areas were depleted in ^{15}N and enriched in ^{13}C relative to their respective control areas. The δ^{15}N of opportunistic algae including *U. intestinalis* generally reflects that of its N source with negligible fractionation (Cohen and Fong 2005). Thus, the macroalgal results may be compared with the results of a comprehensive review of the nitrogen isotopic signatures of nitrate sources by Xue et al. (2009) (see Figure 1-3, Chapter 1). The isotopic signatures of algae from both the SGD areas (~6.8 and 7.1 ‰) and control site (~7.6 ‰) fall within the reported range for groundwater which is contaminated with nitrate sources (~3 to 10 ‰) (Xue et al. 2009). The control area sample (~7.6 ‰) falls within the reported range for surface water which contains nitrate sources (~7 to 11.5 ‰) (Xue et al. 2009), while the SGD areas samples (~6.8 and 7.1 ‰) are largely below this range.

The depleted ^{15}N isotopic signature of the SGD food web components compared to the control suggests that the SGD food web received a nitrogen contribution from a source depleted in ^{15}N. The $\delta^{15}N$ of fertilizer and atmospheric deposition usually ranges from - 5 to + 5 ‰ (average around 0 ‰), while nitrogen derived from human/animal waste usually ranges from + 10 to + 20 ‰, but can be higher depending on the type and level of prior treatment (Xue et al. 2009, Chapter 1; Figure 1-3). In marine systems, $\delta^{15}N$ usually varies between + 5 and + 15 ‰ with an average of ~+ 10 ‰ (Kendall et al. 2007). A study of the stable isotopic signature of nitrate in the groundwater of the aquifers underlying Ria Formosa catchment, designated a Nitrate Vulnerable Zone under the EU Nitrates directive (91/676/EEC), found $\delta^{15}N$ values ranging from + 4.6 ‰ to + 6.7 ‰ (Stigter et al. 2011). The effect of atmospheric deposition is generally felt close to large industrial areas or regions in their path of trade winds. In agricultural areas with relatively restricted human populations, decreased N isotopic signatures usually indicates synthetic fertilizer application. Given these bounds, Stigter et al. (2011) concluded fertilizer to be the dominant source of nitrogen to groundwater in the system, with septic tank waste possibly an important point source contributor. The average nitrogen isotopic signature of algae at SGD areas was + 6.75 ‰ (± 0.09 ‰) for S4 and + 7.08 ‰ (± 0.26 ‰) for S1. Though at the upper end of the range, these values are in agreement with those documented for groundwater in the region by Stigter et al. (2011), possibly indicating (a) a fresh groundwater component of SGD, and (b) a fertilizer and to a lesser extent septic tank waste nitrogen source in the freshwater portion of the SGD. These values are at the high end of the range for groundwater N in the region. This may be due to the effects of fractionation processes during transit, such as denitrification, which results in enriched ^{15}N of the substrate, in this case groundwater nitrate. Another theory might be that the increased algal isotopic signature might reflect some use of marine water nitrate which might be relatively enriched in ^{15}N. However, the amount of N sequestered by the algae from the lagoon marine water upon tidal influx is expected to be negligible due to very low water column N concentrations compared to those previously documented in the groundwater in the region (up to 5500 µM) (Stigter et al. 2011).

The trophic transfer of energy and nutrients between the two trohpic levels which were sampled is relatively indirect. *Ulva sp.* is a primary producer, and *B. reticulatum* is an infaunal omnivore which relies on both herbivory and detritivory (Borja 1986; Gacia et al. 2009). *B. reticulatum* consumes microphytobenthos and other plant detritus found within the sediment. As these two components are not direct steps or links in the food web, they provide two distinct indicators of the food web isotopic signature.

The lower average $\delta^{15}N$ signature of *B. reticulatum* at the SGD relative to control areas might be explained by (1) ingestion of infaunal rather than surface organic matter; (2) selective ingestion of particular food sources at SGD areas; or (3), reduced food availability at control areas. Infaunal primary producers (microphytobenthos) at the SGD sites may assimilate groundwater-borne N. Where the groundwater-borne nitrate is ^{15}N depleted, this will lead to ^{15}N depletion of microphytobenthos. Also, *B. reticulatum* may preferentially predate on diatoms, microbes or select microphyte species which may have increased abundance due to the favorable physicochemical conditions caused by the presence of SGD. These diatoms, microbes and microphyte producers may fractionate during nitrate assimilation, particularly under elevated nitrate concentration, leading to further ^{15}N depletion (Waser et al. 1998; Granger et al. 2004). These processes link the isotopic signature of *B. reticulatum* to that of the elemental nitrogen at the base of the food web and ultimately the source of elemental N to the system.

The current study did not investigate the microphytobenthic community. Microphytobenthos (MPB) can account for a significant portion of total primary production in estuaries, shallow-water marine habitats and soft sediment intertidal environments (Wilson 2002; MacIntyre et al. 1996). MPB can contribute in excess of 25 % of total production in some systems (Colijn and de Jonge 1984; Wilson 2002), with production values reported from the U.S. and Europe generally ranging from ~80 $gCm^{-2} y^{-1}$ to 150 $gCm^{-2} y^{-1}$ (Colijn and de Jonge 1984). The role MPB in production in the Ria Formosa Lagoon and the patterns and causes of MPB spatial and temporal variability in the Lagoon have been investigated (Brito et al. 2010; Brito et al. 2009). Microphytobenthic chlorophyll *a* in the lagoon was measured during 2006, and from 2007 to 2008 (Brito et al. 2009; Brito et al. 2010). Brito et al. (2010) estimated a MPB biomass of 269 mgm^{-2} for the whole lagoon. Brito et al. (2010) found that benthic microalgal biomass was large relative to phytoplankton biomass, with MPB accounting for ~99 % of total microalgal production in the system. Thus the microphytobenthic community is an important and significant component in this system (Brito et al. 2010). Brito et al. (2009) anticipated a seasonal pattern of MPB biomass as the literature reports a positive relationship between MPB biomass and both temperature and irradiance (Colijn and de Jonge 1984). However, seasonal variation could only explain ~5 % of the estimated total variance in MPB biomass (Brito et al. 2009) with no clear patterns of variation or seasonal peaks for benthic chlorophyll *a* concentrations from 2006 to 2007. These researchers concluded that variation in MPB biomass in the lagoon is driven by other factors which have a higher annual frequency than the seasonal effect (Brito et al. 2009). Further, no correlation was found between MPB biomass and tidal range, solar irradiance, salinity, water nutrient concentrations (Brito et al. 2009) or porewater nutrients (Brito et al. 2010), leading these researchers to conclude that MPB variability is a result of a complex interaction of factors (Brito et al. 2009). In terms of spatial distribution, MPB biomass was not homogenously distributed throughout the lagoon and displayed large spatial variability (Brito et al. 2009). Benthic microalgae can be extremely variable in space at scales from centimeters to kilometers (Brito et al. 2009) influenced by factors such as sediment type, and wave and nutrient dynamics (Brito et al. 2009). Brito et al (2009) concluded that most of the variability in MPB biomass was due to small and large scale heterogeneity.

The influence of SGD over the microphytobenthic community has not yet been studied for any system. Due to the effects of light limitation, the highest rates of microphytobenthos production occur when the sediment is exposed, rather than covered with water (Colijn and de Jonge 1984). Thus, the effects of SGD on microphytoplankton growth may be inhibitory due to the constant submersion, or beneficial where associated with increased nutrient supply. It is likely that the actual effects of SGD on microphytobenthos growth will be set by a dynamic balance between these and other counteracting forces, and modulated by other factors. The effect of SGD on microphytobenthos production in the Ria Formsa lagoon is an area for future research. SGD in the Ria Formosa Lagoon may be for one of the highly variable factors accounting for at least some of the spatial and temporal variation in MPB biomass documented by Brito et al. (2009) and Brito et al. (2010).

The rate of tissue turnover (i.e. replacement of new tissue with old) will also affect organism isotopic signature. Isotopic fractionation and isotope turnover rates are not uniform among tissues within an individual, or among species with different physiologies (Filgueira and Castro 2010). Therefore, although SIA provides long-term information about a consumer's diet, this integrative time frame varies from days to weeks according to the species, their growth rate and where relevant, the tissue considered (Filgueira and Castro 2010). Furthermore, organism δ^{15}N values may vary due to metabolic factors including nutritional stress, weight change, life stage and starvation (Fertig et al. 2010). It is possible that the altered isotopic signatures of *B. reticulatum* observed between the SGD and control areas are the result of altered stable isotope turnover rates between the areas, possibly induced at least in part by the presence of SGD and its effects on the primary production and food web metabolism. For example, starvation/reduced food availability is associated with reduced apparent fractionation of N and resultant increases in consumer δ^{15}N values (Hobson et al. 1993). Reduced primary production at the control relative to SGD areas may have elevated consumer elevated δ^{15}N values by limiting the availability of primary food sources.

Algae and *B. retticulatum* at the SGD areas were enriched in ^{13}C relative to control areas. The δ^{13}C value of algae from control areas was –17.22 ‰, which is in very good agreement with the reported average δ^{13}C of marine benthic algae, ~–17 ‰ (France 1995). The δ^{13}C value of algae at SGD areas was significantly higher, by ~2 ‰. Two possible explanations are suggested for this: (a) increased metabolic and respiratory rates at SGD areas, or (b) the carbon contained in the freshwater component of the SGD had an elevated δ^{13}C signature.

The δ^{13}C of total dissolved inorganic carbon (DIC) in the ocean ranges from 0 to 2 ‰ (Zeebe and Wolf-Gladrow 2001, p.168), while that of groundwater can be relatively lower, normally ranging from - 17 to 0 ‰ (Zeng and Masiello 2008; Dorsett et al. 2011; Spiker 1980). The main sources of carbon to freshwater are atmospheric CO_2; dissolution of carbonates by carbonic acid, sulphuric acid and nitric acid in rainwater

(Brunet et al. 2005); and respired CO_2 (Kendall et al. 2001). All three sources provide DIC with low $\delta^{13}C$ values. The $\delta^{13}C$ of atmospheric CO_2 ranges from - 14.4 to - 6 ‰ (Górka et al. 2011; Brunet et al. 2005). DIC produced from the dissolution of carbonates generally results in $\delta^{13}C$ which ranges from - 15 ‰ to 0 ‰, but it is usually ~0 ‰ (Brunet et al. 2005). Carbon released from algae due to respiration usually reflects the signature of the algal carbon source (Kendall et al. 2001), which can range from - 3 to - 35 ‰, but averages - 17 ‰ (Maberly et al. 1992; Raven et al. 2002; Raven et al. 1995; Vizzini and Mazzola 2003). Some fractionation also occurs during microbial remineralisation of fixed carbon (Michener and Lajtha 2007, p.69). Remineralisation of organic matter releases DIC which is depleted in ^{13}C, initially at least, until the organic substrate is nearly completely consumed. When the substrate is nearly completely consumed, the DIC formed is enriched in ^{13}C until all the substrate has been converted to product, at which point there is no apparent net fractionation and the DIC formed has an isotope signature of ~-17 ‰ (Maberly et al. 1992; Raven et al. 2002; Raven et al. 1995; Vizzini and Mazzola 2003). Thus the freshwater DIC is depleted in ^{13}C relative to marine water, and freshwater displays lower $\delta^{13}C$ values than marine water. Due to the reduced $\delta^{13}C$ values of freshwater relative to marine water, food web utilisation of a fresh groundwater DIC source, though it may not lower organism $\delta^{13}C$ values any further, would not result in an increase in organism $\delta^{13}C$ values, as was observed in the current study. Thus, this theory is rejected.

Algal ^{13}C enrichment is inversely proportional to the concentration of DIC, and largely CO_2, available (Fogel et al. 1992). Algal ^{13}C enrichment may reflect either decreased DIC supply or increased DIC demand due to increased algal respiration and production (Sharkey and Berry 1985). In this study, it is suggested that algal ^{13}C enrichment at SGD areas is due to an enhanced rate of carbon turnover caused by enhanced community respiration due to nitrogen inputs from (a) fresh groundwater, and/or (b) enhanced mineralisation. The relative role and importance of the two sources ((a) and (b)) remains to be determined. The recirculated seawater compartment of SGD can strongly enhance reaction rates in the benthic permeable sediments and nitrate fluxes in discharging waters (Ibánhez et al. 2012); this enhanced mineralisation mechanism has been described for this site (Ibánhez et al. 2012). Increased respiration of a benthic intertidal community in an area of SGD has been described elsewhere through measurement of CO_2 exchange during light and dark phases (Migné et al. 2011a).

The tissue nutrient contents (%C and %N) of macroalgae and *Bittium reticulatum* did not differ between SGD and control sites. Similarly, algal C:N ratios did not differ between SGD and control sites. *Ulva* spp. % C and % N were relatively low compared to other literature reported values, e.g. 26 ± 7 %C and 3 ± 0.7 %N reported by Faganeli et al. (1988), however the C:N ratios are in line with those reported elsewhere. The C:N of *Ulva* spp. normally ranges from 7 to 25 (Viaroli et al. 2005; Wheeler and Björnsäter 1992; Faganeli et al. 1988). The lower than expected absolute carbon and nitrogen content may be due to reduced growth in winter. The critical C:N ratio of *Ulva* spp., i.e. that required for balanced growth, is ~9.6 (Viaroli et al. 2005). C:N ratios in excess of this may indicate potential N limitation, and ratios below this, potential N loading. In general, algal C:N ratios across all sites are similar to those reported for non-eutrophic sites (Faganeli et al. 1988; Wheeler and Björnsäter 1992) and do not indicate excessive nitrogen loading.

The C:N ratio of *Bittium reticulatum* tissue differed between the SGD and control area on beach face section B. The ratio was elevated at the SGD relative to the control area (6.18 ± 0.76 vs. 4.66 ± 0.13). Predation on food sources which have a higher C:N ratio than required by the consumer leads to decreased consumer growth efficiencies for C (Vrede et al. 2004). This eventually leads to low consumer growth rate and biomass and low grazing pressure on autotrophs (Vrede et al. 2004). Given the lack of consistent effects/statistically significant results in the current study, it is likely that the result observed difference for *B. reticulatum* tissue C:N is not attributable to the presence of SGD. These results indicate the superior efficiency of stable isotope analysis relative to analysis of tissue elemental content in the study of nutrient trohpic transfer associated with SGD in the current system.

SGD was associated with altered structure and composition of a section of the benthic intertidal food web. SGD altered the composition and biomass of the intertidal macroalgae community, similar to the results of Migné et al. (2011). SGD was associated with opportunistic green macroalgal species, as was also found for intertidal SGD in France (Ouisse et al. 2011) and subtidal SGD in Cape Cod, USA (Valiela et al. 1990). The proliferation of such freshwater-tolerant opportunistic green macroalgae in otherwise marine environments may be used as an indicator of freshwater input and nutrient additions, possibly derived from SGD in some systems. SGD was associated with altered structure and composition of the non-sessile macroinvertebrate community. This corroborates the findings of previous work which documented changes in species abundances associated with intertidal SGD in Cape Cod (Dale and Miller 2008; Miller and Ullman 2004), the German Wadden Sea (Zipperle and Reise 2005) and Roscoff Bay in France (Migné et al. 2011b). Similar to the results of the current study, Migné et al. (2011) found no effect of SGD on the total macroinvertebrate biomass, indicating the differing effects of SGD on species diversity, abundances and biomasses.

This is only the second time that SIA of organism tissue has been used in the study of food web dynamics associated with intertidal SGD. Similar to the previous study by Ouisse et al. (2011) the current study found that the $\delta^{13}C$ and $\delta^{15}N$ values of food web components differed between SGD and control areas though for both studies the results were somewhat ambiguous and were not applicable across all sites. Though further research is necessary the current study and that of Ouisse et al. (2011) suggest that SIA may provide a useful tool for identifying two separate food webs in systems in receipt of, one the SGD dependent food web and the other, the control food web. The results herein indicate that SIA may be used to trace SGD-borne N into and through SGD food webs. This research also indicates that carbon isotopic signatures may inform on alterations to nutrient cycling associated with SGD.

The results of this study may be linked to other less obvious ecological mechanisms underlying the system. For example, stable substrata which remain at the illuminated sediment surface are essential for small propagules to germinate (Schories et al. 2000). Surface-dwelling mobile macrofauna such as snails serve as germination substrata for algal propagules (Schories and Reise 1993; Schories 1995). A positive feedback loop may exist between recruitment and development of both macroalgal spores and shelled herbivorous and detritivore macrofauna. The significance of Hydrobiid snails for *Ulva* spp. germling recruitment has been investigated for the Ria Formosa and five other comparable locations by Schories et al. (2000). This positive feedback loop occurs in the Ria Formosa, but is less significant for the establishment of algal mats than overwintering of adult fragments and secondary drift import of adults (Schories et al. 2000). The presence of SGD, however, might modulate the importance of this process. Schories et al. (2000) noted that periods where adult macrophyte coverage was absent were very short in the Ria Formosa and the lagoon didn't display the same seasonality of macroalgae cover and development apparent at the other five locations (Schories et al. 2000). This anomaly may be connected to the presence of SGD, as SGD may provide favorable growth conditions, which facilitate year round algal production. In this way, SGD may aid in initiating and maintaining the macroalgal blooms experienced in the lagoon, particularly those blooms which occur in winter. Most of the macroalgae blooms developing in the Ria Formosa to date have occurred during winter and the initial development of these blooms always occurred in the sandy beach habitat (Aníbal 2004).

Organism depletion in ^{15}N at SGD areas suggests a synthetic fertilizer nitrogen source. Is it proposed that organisms at the SGD sites were enriched in ^{13}C due to increased rates of carbon turnover, respiration and primary production, congruent with the ecological results of increased macroalgal and macroinvertebrate biomass at SGD areas. When occurring, SGD seepage in the Ria Formosa can carry nitrogen at concentrations up to 187 µM (Leote et al. 2008). Leote et al. (2008) hypothesized that SGD might modulate primary production and support the seasonal nuisance algal blooms which are a major source of primary production in the system (Sprung 1994). The results presented herein support this theory. In this system, SGD modulates both community composition, and nutrient (particularly carbon) turnover. SGD is a significant agent in food web structure and trophic transfer, in this case on a small/medium spatial scale, but possibly also on larger spatial scales in more heavily SGD areas. The carbon and nitrogen isotopic signatures of surface estuarine macroalgae and macroinvertebrates vary temporally (Claudino et al. 2013). In the current study all sampling was conducting in the same one season, and thus any effect of seasonality would be expected to apply across all sites studied and would not account for any inter-site isotopic variation. However, seasonal variation in isotopic signatures of lagoon organisms may present an area for future research.

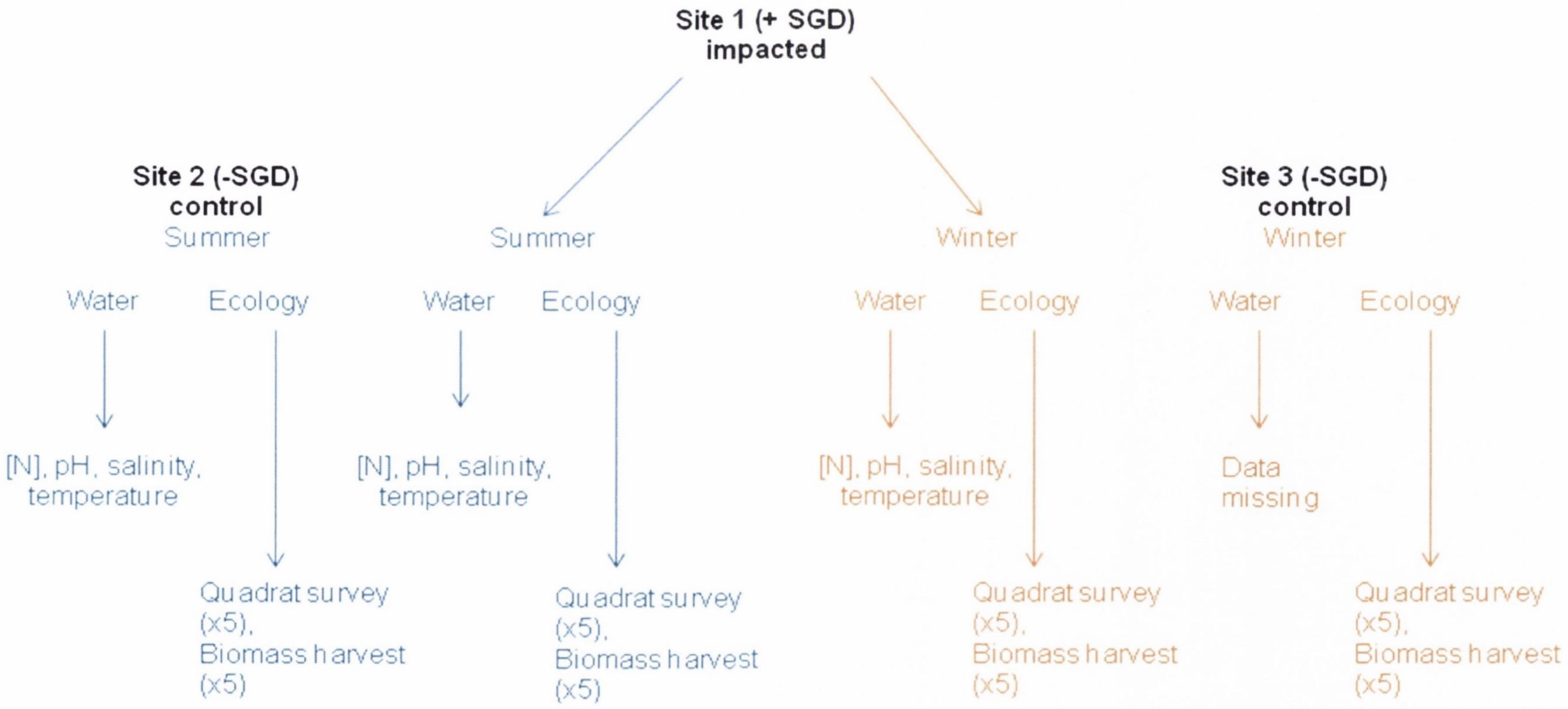
Chapter 3. The ecological impacts of karst-channelled intertidal freshwater submarine groundwater discharge (SGD) on the rocky intertidal macroalgal and macroinvertebrate community; Ireland.

3.1. Abstract

Intertidal discharge of fresh groundwater occurs worldwide, but is particularly noticeable along karst coastlines. Submarine groundwater discharge (SGD) induces measureable changes in temperature, salinity, pH and nutrient concentrations in receiving systems, however little has been done to assess the ecological impact of these changes over intertidal communities. In the first study to investigate the ecological effects of karst-channeled intertidal SGD on a rocky shore community, one SGD and one control site were surveyed during summer 2011 and winter 2012, in Kinvara bay on the west coast of Ireland. Salinity, temperature and nitrogen concentrations were analysed in groundwater, SGD and bay water. Groundwater and SGD were similar in salinity, and this salinity was lower than that of the marine compartment. The temperature of SGD and groundwater also differed from that of the marine compartment. In summer, SGD contained elevated nitrogen concentrations (~50 µM) compared to control site water samples (~16 µM), while SGD sampled in winter had nitrogen concentrations ~8 x that found in marine water (~85 µM v. ~10 µM). The presence of SGD was associated with altered percentage cover of motile organisms ($p < 0.05$) and greater cover of *Fucus ceranoides* (75.5 ± 4.8 % in summer, and 86.2 ± 4.0 % in winter) relative to the control sites (47.1 ± 4.8 % in summer, and 43.3 ± 4.2 % in winter). Lower % bare substrate was recorded at the SGD site (4.9 ± 2.0 % in summer, and 7.6 ± 3.0 % in winter) relative to the control sites (13.5 ± 2.1 % in summer, and 40.2 ± 10.6 % in winter). The control sites displayed greater cover of

the opportunistic green macroalgae *Ulva* spp. in summer (31.8 ± 7.0 %), and *Cladophora rupestris* in winter (16.5 ± 7.3 %) relative to the SGD site (3.3 ± 0.5 % for *Ulva* spp. in summer, and 7.6 ± 3.0 % for *Cladophora rupestris* in winter). SGD sites had increased macroalgal biomass (58.66 ± 11.61 g/0.5m^2 in summer, and 129.79 ± 17.75 g/0.5m^2 in winter) relative to the control sites (13.60 ± 3.53 g/0.5m^2 in summer, and 20.03 ± 11.97 g/0.5m^2 in winter) ($p < 0.05$ in both seasons). SGD sites had an altered macroinvertebrate assemblage in terms of species richness and abundances of individual species compared to the control site ($p < 0.05$ in both seasons). The macroalgal and macroinvertebrate community at the SGD site differed between seasons ($p = 0.02$ and $p = 0.008$ resp.), with greater algal biomass in winter (128.37 ± 16.83 g/0.5m^2) than summer (58.65 ± 11.60 g/0.5m^2) ($p = 0.03$) and greater macroinvertebrate abundance, largely composed of *Jaera albifrons* in winter (915.4 ± 426.1 individuals per 0.5m^2) than summer (72.0 ± 19.2 individuals per 0.5m^2) ($p = 0.008$). Sessile species percentage cover at the SGD site also differed between seasons ($p = 0.008$). Overall, the results indicate that (a) karst-channeled intertidal freshwater SGD is (i) associated with a different physicochemical makeup in terms of nitrogen concentration, salinity and temperature relative to that of the receiving marine environment, (ii) temporally and spatially alters community structure and composition, and finally, (b) the physicochemical characteristics of SGD, induce, at least in part, the observed SGD associated ecological modifications.

3.1.1. Flow chart of Chapter 3 Experimental Design and Structure



Flow chart indicating samples collected and analysis undertaken for SGD site 1 and control site 2 in summer (blue), and SGD site 1 and control site 3 in winter (orange). Water samples were also taken for nitrogen concentration analysis and water chemistry analysis from the same one inland groundwater borehole (GW) in summer and winter, and from Galway bay (GB) in winter.

3.2. Introduction

Submarine groundwater discharge (SGD) is defined as all water crossing the seabed into the coastal ocean, regardless of fluid composition or driving force (Burnett et al. 2003), over a scale length of meters to kilometers (Moore 2010). SGD may consist of three water types: recirculated seawater, fresh groundwater, or a brackish mixture of both (Burnett et al. 2003). SGD can deliver large fluxes of freshwater to marine environments, and is estimated to be responsible for as much as 6 – 10 % of total global oceanic freshwater inputs (Taniguchi 2002), hence providing an important pathway for terrestrial nutrients and other dissolved species to the marine environment (Slomp and Van Cappellen 2004). Discharging water can differ from the receiving water body in a number of physicochemical properties including temperature, salinity, and concentrations of macro- and micro- nutrients, as well as other contaminants. Some of these differences occur naturally (e.g. temperature and salinity) while others are the result of anthropogenic activity (e.g. loading of macro- and micro-nutrients, pesticides, organic compounds and microbial pathogens). Therefore, while SGD is a natural phenomenon which can in itself lead to altered ecosystem functioning, it becomes of concern when it is fuelled by water contaminated due to anthropogenic activities.

Nutrient enrichment in the marine environment (particularly of nitrogen, normally the limiting nutrient in marine systems (Howarth and Marino 2006; Nixon 1995)) can increase primary production. Enhanced primary production (i.e. eutrophication), if sustained, eventually produces negative community and ecosystem level impacts driving changes in species composition and affecting upper trophic levels of the food web (Valiela et al. 1990). This phenomenon, observed worldwide (Howarth et al.

2000), constitutes a major threat to the balanced functioning of aquatic systems (Diaz and Rosenberg 2008) by inducing deleterious effects on the sustained provision of ecosystem goods and services (Worm et al. 2006). Diffuse SGD has been linked to increased primary production (Miller and Ullman 2004; Waska and Kim 2010b), red-tide development (Lee and Kim 2007), the onset and development of eutrophication (Dong-Woon Hwang et al. 2005; Valiela et al. 1990) and harmful algal blooms (Paerl 1997) and changes to species diversity (Kamermans et al. 2002).

SGD is frequently found where karst aquifers are hydraulically connected to the sea (Fleury et al. 2007; Slomp and Van Cappellen 2004). Pathways created by limestone dissolution allow rapid infiltration and relatively unrestricted conduit flow of groundwater in karst aquifers (Coxon and Drew 2000, p.20). These natural pathways provide focused, well defined coastal entry points rendering karst-channeled SGD a 'point' rather than 'diffuse' mode of delivery of groundwater borne solutes from land to sea. Large volumes of freshwater SGD can be rapidly delivered to the coast via these point entries (Drew 2008), reducing the marine water dilution capacity in the vicinity of discharge locations. Also, low aquifer residence time and normally well-oxygenated conditions reduce the capacity for nutrient mitigation and removal during transit (mostly via denitrification in the case of N). Furthermore, fast conduit water flow generally results in a short time lag between application of pollution at source (soil leaching, septic tank leakage, etc.) and discharge to the sea. Due to these particular hydrogeological conditions of karst aquifers, karst-channeled point-source SGD is expected to induce more acute ecological change than diffuse SGD.

To date, the majority of SGD-related research has focused on quantifying the fluxes of water (particularly freshwater) and estimating associated mass delivery of dissolved/colloidal constituents. The ecologically relevant abiotic factors which naturally vary with SGD and the ecological alterations associated with both natural and anthropogenically-impacted SGD have however received little attention and remain largely un-quantified (Moore 2010). Those studies which directly concern the ecological changes associated with intertidal SGD are confined to a small number of geographical locations (McClelland and Valiela 1998; Migné et al. 2011b; Ouisse et al. 2011; Silva et al. 2012; Zipperle and Reise 2005; Kotwicki et al. 2013), and sometimes investigate the ecological effects of SGD that is fed by an aquifer by a significant, discrete pollution event, on a select sample of indicator algal species only (Carruthers et al. 2005; Mutchler et al. 2007; Kamermans et al. 2002).

Of the few existing studies concerning the ecological impacts of SGD, only two focus on karst-channeled intertidal SGD, both in the Yucatan peninsula, Mexico (Mutchler et al. 2007; Carruthers et al. 2005). Both studies consider the effect of a known pollutant (wastewater) conveyed via SGD on select seagrass species. More recently, the ecological impact of karst-channeled subtidal SGD on the meiofaunal assemblage was studied in southern Portugal (Encarnacão et al. 2013). This study found no significant effect of SGD; however, the ecological effects of subtidal SGD are expected to differ from those of intertidal SGD due to differences in marine water dilution capacities in these two environments. Currently, no published literature documents the effects of karst-channeled SGD on the intertidal macroalgal and macroinvertebrate assemblage, or the seasonal effects of this type of SGD on intertidal community structure and composition.

Where land limestone formations meet the sea, rocky intertidal systems provide substrate for colonisation and succession of macroalgae (Sousa 1979b), supporting subsequent trophic levels and maintaining healthy ecosystem functioning. Macroalgae and rock crevices provide shelter and refuge for invertebrates (Coull and Wells 1983) and fish species, and are important feeding, resting, spawning and nursery habitats which underpin many socioeconomically relevant ecosystem goods and services (Thompson et al. 2002). Although rocky shores are ubiquitous along the coastline and despite their relevance in terms of ecosystem functioning and associated socioeconomic importance, they are relatively understudied (Araújo et al. 2006; Atalah and Crowe 2010; Boaventura et al. 2002; Caceres-Martinez et al. 1993).

The aims of the research described in this chapter were to (1) determine if the macroalgal and motile macroinvertebrate assemblage differed (a) between SGD and control sites, and (b) at the SGD site between seasons, (2) determine if SGD temperature, salinity, pH and nitrogen concentrations differed from that of marine compartments, and (3) consider how the former relate to and structured the later.

To address these aims, the following alternative hypotheses were proposed (Ha) and associated null hypotheses (Ho) tested:

(1)

Ha: The community composition and biomass of harvested macroalgae will differ between SGD and control sites.

Ho: There will be no difference in the community composition and biomass of harvested macroalgae at the SGD sites and control sites.

(2)

Ha: The structure and composition of the motile macroinvertebrate assemblage, assessed in terms of species type and abundance, will differ between SGD sites and control sites.

Ho: There will be no difference in the structure and composition of the motile macroinvertebrate assemblages, assessed in terms of species type and abundance, at the SGD sites and control sites.

(3)

Ha: The community structure and composition, assessed in terms of percentage cover of non-motile/attached species, will differ between SGD sites and control sites.

Ho: There will be no difference in the community structure and composition, assessed in terms of percentage cover of non-motile/attached species, between SGD sites and control sites.

Furthermore, the results of the above hypothesis tests are discussed in light of the measured nitrogen concentrations, salinity, pH and temperature of SGD and marine compartments to postulate how the former might relate to and structure the later. AZTI's marine biotic index (AMBI) and the multivariate AMBI (M-AMBI) are computed to further characterise the influence of SGD over the ecological health of the system and assess the role of SGD as a disturbance in the system.

3.3. Methods

3.3.1. Study area

The study site, Kinvara bay, is situated on the mid-western coast of Ireland (53° 8' 22.79" to 53° 10' 35.08" N and 008° 56' 10.34" to 008° 57' 50.45" W). The bay is ~4 km in length and 2 km at its widest point, with over half the length of the bay less than 1 km wide. It is a smaller inlet of the larger Galway bay (53° 14' 5.32" to 53° 7' 54.99" N and 008° 58' 64.00" to 009° 34' 22.00" W) (Figure 3-1). The tidal regime is mesotidal with 3 – 5 m tidal range in the bay (Smith and Cave 2012). There is a well documented locus of intertidal discharge at the head of Kinvara bay (Wilson and Rocha 2012; Drew and Daly 1993; Drew 2008). The karst aquifer which feeds SGD into the bay is underlain by Carboniferous limestone bedrock (O'Connor et al. 1993). Mean annual precipitation ranges from 1000 – 1400 mm, with monthly precipitation ~150 mm in winter and ~50 mm in summer (Kiely 1999). Approximately half of this precipitation becomes groundwater recharge (Drew, 2008). With an absence of noticeable surface flow, all water for over 500 km^2 moves through underground caverns and conduits to Galway bay (Drew and Daly 1993) where SGD discharges at an estimated 5 - 30 m^3 s^{-1} (Drew 2008). This rate of discharge is high relative to other literature reported values which normally range from 0.6 to 35 m^3 day^{-1} (Leote et al. 2008; UNESCO 2004; Boehm et al. 2006). Salinity within Kinvara bay ranges from 0 - 32.9 psu and temperature from 11.2 °C to 16.7 °C (Cave and Henry 2011). Generally, warm summers and mild winters characterise the cool temperate maritime climate (Kiely 1999).

Kinvara village, the main urban agglomeration in the area, is located ~600 to 800 m from the locus of SGD. In 2011, Kinvara village and the surrounding town land of Gort had a combined population of 2,644 (CSO 2011) with 70 % of those living in rural areas (McGovern 2012). The bay is a natural oyster bed (Cannon 2010), the site of mussel aquaculture industry and the focus of recreational activities vital to tourism in the region. Tourism (~18,000 people per season (Failte Ireland 2014)), which is the main tertiary industry in the region provides the main source of employment (Gallagher et al. 2010) and brings a concomitant influx of pollution to the area, transferred into the bay as sewage. An estimated 315 m^3 of untreated sewage enter Kinvara bay through a point source outflow pipe every day (Cannon 2010, p.166). The sewage flow pipe which discharges at the intertidal is exposed a low tide and covered at high tide. Agriculture is the main primary industry in the region (Gardiner and Radford 1980) with agricultural land used mostly for pasture and non-irrigated arable land for sheep and cattle grazing (EPA 2006). The region has, however, the lowest agricultural productivity in Ireland and is classified as 'marginal' (Crowley et al. 2004). Due to the grassland-dominant low intensity agriculture and a low level of industry and urbanization, groundwater pollution derives mostly from septic tank leaks and effluent from farming activities (Thorn and Coxon 1992). Pollutants include nutrients of eutrophication concern (phosphorus and nitrate), pesticides, antibiotics, and microbial pathogens (Coxon 2012).

3.3.2. Experimental design

Sampling was conducted in summer 2011 (June) and winter 2012 (November). To determine the impact of SGD on intertidal ecology, one SGD and one control site were surveyed and sampled each season. To determine if ecological differences existed at the SGD site on a seasonal basis, the same SGD site (site 1) was sampled during both seasons, whereas different control sites were sampled between seasons (site 2 and 3). In both seasons, ecological quadrat surveys were conducted to address null hypothesis (3), and algal and macroinvertebrate biomass sampling were carried out to test null hypotheses (1) and (2) respectively (*n* = 5). Water salinity, pH and temperature were recorded, and samples were taken for determination of nitrogen concentration. Fieldwork was carried out and all samples collected over a two day period in each season (Figure 3.1.1.).

3.3.3. Selection of study sites

Site 1 (common to summer and winter) was located at the entry point of SGD into the bay. In summer, the control site (site 2) was located to the west of site 1 and the control site in winter (site 3) to the east on the far side of a rocky outcrop (Figure 3-1). Sites 2 and 3 distanced ~300 and ~100 meters, respectively, from site 1. Site 1 was chosen based on the presence of focused SGD, previously documented (Drew 2008). Site 2 and 3 were chosen for their close proximity to site 1 to reduce variation in external factors (as per Kotwicki et al. 2013; Silva et al. 2012; Migné et al. 2011b; Ouisse et al. 2011). At low tide, site 3 was completely exposed to the atmosphere while trickles of water of reduced salinity (12 psu) were a feature of some sections of site 2. The sewage outflow pipe is located ~750 meters to the west of site 1 (Figure 3-1). Site 2 distanced ~450 m from the sewage outflow pipe, and site 3 distanced ~850 m from the outflow pipe. It was desirable for site 2 to be distant from this pipe of waste water, while also located on the same side of the bay as site 1 to maintain comparability of sites.

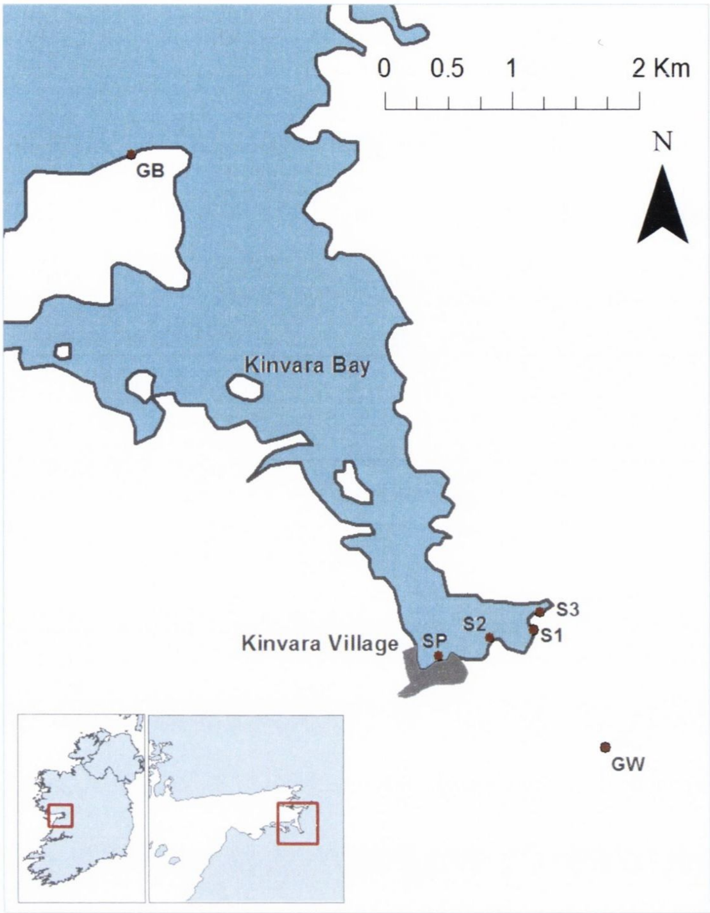


Figure 3-1 Study site and sample locations. Galway Bay and smaller inlet, Kinvara bay, indicating sampling locations; Galway bay (GB), SGD site (S1), and summer (S2) and winter (S3) control site. Also indicated are the locations of the inland groundwater sample (GW) and sewage outflow pipe (SP).

3.3.4. Water sampling and *in-situ* parameters

Water samples were collected from opposite ends of site 1 at low tide in both seasons (samples SGD(S1)a and SGD(S1)b in summer; SGD(S1)c and SGD(S1)d in winter), site 1 at high tide in summer (SGD(S1)ht), an inland groundwater borehole (53° 07' 36.65" N 008° 55' 08.42" W) located ~1 km from the site of SGD in both seasons, the control (S2) in summer, the marine environment associated with site 1 and site 2 at ~15 cm depth of water in winter (samples 'SGD/marine' and 'control/marine' respectively), and Galway bay at the mouth of Kinvara bay in winter. Samples were collected in 500 ml or 1000 ml acid-washed PE bottles. One PE bottle was taken for each sample and from this 2 - 4 individual replicate water samples filtered. With the exception of site 2 where water depth was insufficient, all the samples were collected by immersing the polyethylene (PE) bottles closed, and subsequently opening them underwater to draw the sample. Water was immediately filtered through poly-ether-sulphone (PES) membranes (Rhizon SMS-10 cm; Eijkelkamp Agrisearch Equipment 0.1 µm pore size) into sterile 11 ml vacuum tubes (BH vacutainer) via a needle connected to the membranes with Tygon tubing. The first 5 ml of each sample was discarded as dead volume. Collection by this method precludes the need for further preservation (Luo et al. 2003; Seeberg-Elverfeldt et al. 2005). Samples were stored at 4°C until analysis. Water samples were analysed for combined NO_3^- and NO_2^- via flow injection analysis (FIA) on a Lachat^{TM} QuickChem 8500 instrument following the dual determination of nitrite and nitrate via flow injection analysis using a cadmium column method (Anderson 1979; Johnson and Petty 1983). Salinity, pH and temperature were measured using an in-situ WTW Cond197i multi parameter probe and GPS location recorded with a Garmin^{TM} eTrex handheld GPS navigator.

3.3.5. Quadrat survey

Quadrat surveys were conducted in order to test null hypothesis (3). To ensure maximum exposure of the sites, sampling was conducted during low spring tide. At each site, a 5 m^2 area study station was delineated. As there was no visible vertical shoreline zonation, all stations were located at approximately the same shore height as determined by measuring the distance from the seaward end of the sampling station to the water at low tide (~25 m). Point intersection quadrat surveys were used at all sites.

The 5 m^2 area was subdivided into 0.5 m^2 units and random number tables were used to determine the location of five 0.5 m^2 quadrats at each site. The 0.5 m^2 quadrats were strung with robust twine to create intersections every 1 cm. Sampling was stratified by layers, i.e. where one living species overlay another, all living species were recorded. Quadrats were double strung to remove the compulsion to correct the path of the needle to account for perceived change in location/direction of the needle due to parallax error upon moving from air to water. Organism identification was done *in-situ* when possible, but in the case of taxonomic uncertainty a sample was collected and identified in the laboratory. Percentage cover data was calculated for species or substrate per quadrat as:

$$\frac{NIH_s}{\sum NIH} \times 100$$

where NIH_s is the number of intersection hits for the relevant species or substrate and ΣNIH the total number of intersection hits for the quadrat.

3.3.6. Biomass survey

Biomass surveys were conducted to address null hypotheses (1) and (2). For each quadrat, a 15 cm^2 hollow sub-quadrat was used to delineate an area from which all biomass was harvested using a plastic scraper. Harvested biomass was placed in Ziploc™ bags and transported to the field laboratory on ice. On arrival at the field laboratory, samples were rinsed in a 1 mm sieve to remove smaller particles and organisms, and the remaining sample sorted through. All visible algae and macroinvertebrates were removed and identified to the lowest possible taxonomic level using a dissecting scope. Macroinvertebrates were subsequently counted and algae placed in labeled Ziploc™ bags, frozen and returned to the main laboratory, where wet weight biomass was determined using an electronic balance (± 0.01 grams) following thawing.

3.3.7. Data analysis

Non-parametric statistics were used for all analysis to accommodate the small sample sizes (*n* = 5). Multivariate analyses were conducted to relate the presence/absence of SGD, and 'season' for the SGD site between seasons, to sessile organism percentage cover, specific macroinvertebrate taxa composition/abundance, and algal composition/biomass while incorporating the large number of variables (species). ANOSIM (analysis of similarity, R test statistic) was carried out and nmMDS ordinations constructed based on underlying Bray-Curtis derived similarity resemblance matrices (Clarke and Warwick 2001).

ANOSIM is a non-parametric multivariate means of analysis of variance. ANOSIM calculates a *p*-value based on permutations (Anderson 2001). As this is a permutation based method, the maximum permissible *p*-value obtainable is a function of the number of permutations (Anderson 2001). Given the sampling structure of the current experiment with five replicate samples at two sites, the maximum number of possible permutations is 126 as there are 126 ways to make up the five replicates at the two sites where order doesn't matter (i.e. 10!/ (2!(5!)^2)) (Anderson 2001). Where there are 126 possible permutations, the lowest obtainable *p*-value is 0.008 (i.e. 1/126) (Anderson 2001, p.36). Thus, in the current study, the lowest possible *p* is constrained at 0.008. In cases where the *p*-value is 0.008, more information regarding the differences between samples may be gleaned by reference to the magnitude of the associated R value as the value of the associated R test statistic is proportional to the statistical difference, even if the *p*-value is constrained at 0.008.

Non-metric multidimensional scaling (nmMDS) plots are distanced based with the degree and direction of separation indicating the form and degree of difference between samples. A stress value indicates how well the ordination represents the underlying data and is reported for each plot. Stress values of < 0.05 indicate an excellent representation with no prospect of misinterpretation, and values < 0.1 correspond to a good ordination with no real prospect of misleading interpretation (Clarke and Warwick 2001, pp.5–6). Percentage cover data were square root transformed ($\sqrt{}$), and algal and macroinvertebrate data fourth root transformed ($\sqrt{}\sqrt{}$) prior to analysis to reduce skewness.

Univariate Wilcoxon rank sum tests (W test statistic) were used to compare individual parameters. A significance level 95 % ($\alpha = 0.05$) was set for all tests. All multivariate analyses were carried out using PRIMER v6 (Clarke and Gorley 2006) and all univariate statistics were conducted using R (R Development Core Team 2011). The *nortest* package in R was used for Anderson-Darling tests and all other tests were inherent in R and not part of loaded packages.

AMBI and M-AMBI were calculated using AMBI version 5.0 software (AMBI: AZTI MARINE BIOTIC INDEX (AZTI-Tecnalia, www.azti.es)) and the March 2012 species list according to the guidelines from the authors (Borja et al. 2012a). M-AMBI is calculate from AMBI, the Shannon-Wiener index and Richness index and requires a 'Bad' and 'High' reference condition for each of these indices. For the 'Bad' reference condition, these values are always 6, 0 and 0 for AMBI, Shannon-Wieners index and richness, respectively (Borja et al. 2012a). In the current study, the 'relative control' sites are used as the 'High' reference conditions. Thus, the lowest AMBI and highest Shannon-Wiener value and highest richness values from the control sites are used as 'High' reference conditions. These values are automatically selected and input by the AMBI software.

3.4. Results

3.4.1. Water nitrogen analysis and physicochemical parameters

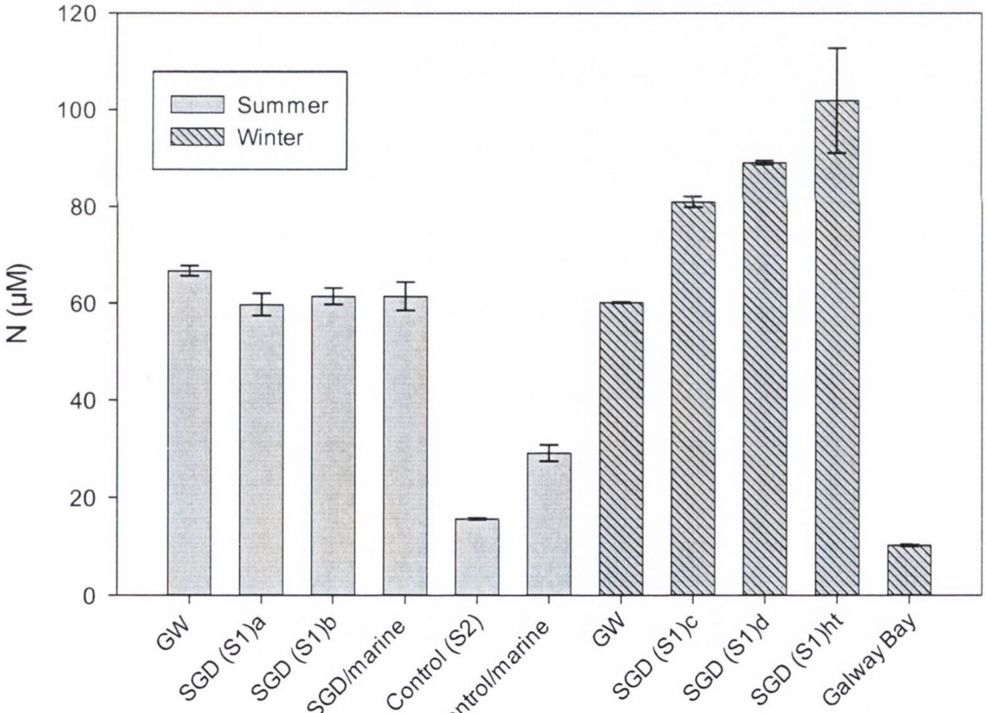


Figure 3-2 Water nitrogen concentrations (NO_3^- + NO_2^-, µM). SGD samples were taken from two points at site 1 at low tide (SGD (S1)a (*n* = 4) and SGD(S1)b (*n* = 3)), the SGD/marine site (*n* = 5), the control (S2) (*n* = 4) and the control/marine site (*n* = 5) in summer. SGD(S1)c (*n* = 3) and SGD (S1) d (*n* = 3) are SGD samples taken at low tide, and SGD(S1)ht (*n* = 3) is SGD sampled from site 1 at high tide, in winter. *n* = 2 for the Galway

bay sample in winter. GW indicates the groundwater sample (*n* = 3 in summer and *n* = 3 in winter).

The nitrogen concentration of SGD in summer was higher than either the control (S2) or control/marine samples (Figure 3-2). The SGD/marine sample had a nitrogen concentration comparable to that of SGD, while that of the control/marine was approximately twice that of control (S2), but half that of SGD and SGD/marine sites (Figure 3-2). In winter, SGD contained nitrogen at concentrations 8 to 10 times greater than Galway Bay water (Figure 3-2). SGD nitrogen concentrations were higher in winter than in summer. The groundwater nitrogen concentration was comparable between seasons. In summer, groundwater nitrogen concentration was comparable to that of SGD, while in winter, groundwater nitrogen concentration was ~20 to 40 μM less than SGD (Figure 3-2).

Table 3-1 Water physicochemical parameters (mean (± SD)). Salinity (psu), pH and temperature (°C) data for both seasons for, where relevant, groundwater (GW), submarine groundwater discharge (SGD (S1)), the SGD/marine site, control site 2 (Control (S2)), and Galway bay. SD values for summer data are based on *n* = 4, and SD values for winter data on are based on *n* = 2. Where no SD is given, values are based on one sample. No water data are presented for the winter control site (control site 3) as no water was present at this site at low tide.

Season	Sample	Salinity (psu)	pH	Temperature (°C)
Summer	GW	0	7.21	16.4
	SGD (S1)	0 (± 0)	7.06 (± 0.2)	11.9 (± 0.3)
	SGD/marine	0	7.07	11.2
	Control (S2)	12.1 (± 1.6)	8.05 (±0.1)	15 (± 0.5)
Winter	GW	0	7.4	11.6
	SGD (S1)	0 (± 0)	7.3 (± 0.2)	11.4 (± 0.1)
	Galway bay	33	8.2	10

All SGD and groundwater samples had salinity 0 psu. The water occurring on some sections of the summer control site had salinity 12.1 psu, and that of the Galway bay sample was 33 psu. In both seasons, the pH of groundwater and SGD was comparable, and lower than that of either the control site 2 or Galway bay water by ~1 unit. SGD temperature was approximately the same (~11 °C) in both seasons. The control (S2) sample was warmer than SGD in summer. Galway Bay water was colder than SGD by ~1 °C in winter (Table 3-1). In winter, the temperature of groundwater was comparable to that of SGD. In summer however, groundwater was ~4 °C warmer than that of SGD.

3.4.2. Sessile community composition

Table 3-2 Percentage cover of sessile species (algae and non-motile macroinvertebrates). Data (mean and 1SE) for SGD site 1 and control site 2 in summer, and site 1 and control site 3 in winter. *n* = 5 in all cases. Brown or red algal species which were unidentifiable in summer are indicated as Unid. spp.1-4.

	Summer			
	Site 1 (+ SGD)		**Site 2 (- SGD)**	
Species	**Mean**	**SE**	**Mean**	**SE**
Fucus ceranoides	75.5%	4.8%	47.1%	4.8%
Ulva linza /Ulva intestinalis	3.3%	0.5%	31.8%	7.0%
Elminius modestus	10.0%	3.3%		
Substrate	4.9%	2.0%	13.5%	2.1%
Ascophyllum nodosum			6.6%	4.2%
Unid. spp. 1	2.8%	1.7%		
Unid. spp. 2	2.7%	1.6%		
Mastocarpus stellatus			1.0%	1.0%
Unid. spp. 3	0.6%	0.6%		
Unid. spp. 4	0.1%	0.1%		
Number of species	8		5	

	Winter			
	Site 1 (+ SGD)		**Site 3 (- SGD)**	
	Mean	**SE**	**Mean**	**SE**
Fucus ceranoides	86.2%	4.0%	43.3%	4.2%
Substrate	7.6%	3.0%	40.2%	10.6%
Cladophora rupestris	7.6%	3.0%	16.5%	7.3%
Elminius modestus	6.2%	2.6%		
Number of species	3		2	

The composition of the sessile/non-motile community (i.e. algae and sessile macroinvertebrates) at the SGD site differed from that at the control sites in both seasons. Thus, null hypothesis (3) of no difference in the community structure and composition between SGD and control sites as assessed in terms of percentage cover of non-motile/sessile/attached species, is rejected. Also, the community composition at the site differed between seasons ($p < 0.05$; Table 3-2). Ten species were recorded in total, nine macroalgae and one barnacle (Table 3-2). *Fucus ceranoides* (Linnaeus, 1753) was the algae and species with greatest coverage at all sites (Table 3-2). Other abundant species were *Ulva.* spp. (Linnaeus, 1753) (~32 % at site 2), *Cladophora rupestris* (Linnaeus, 1753) (~17 % at site 3) and *Elminius modestus* (Darwin, 1854) (10 % at site 1). *Ascophyllum nodosum* (Linnaeus, 1753) accounted for less than ~7 % of coverage at site 2, and all other species accounted for less than 3 % coverage each at any given site. Four species, which accounted for very small percentage cover (< 3 % each) in summer, were unidentifiable. These were either brown or red algae and are indicated as unid. spp. 1-4 in table 3-2. Although *F. ceranoides* dominated coverage at all sites in both seasons, coverage of this species was always greater at the site compared to control sites (Table 3-3). Site 1 was characterised by low bare substrate in both seasons, with twice as much bare substrate at site 2, and over five times as much at site 3 relative to site 1 (Table 3-3). The substrate type at site 1 predominantly comprised large pebbles, cobbles and boulders. The substrate at site 2 comprised of silt, mud, sand, pebbles cobbles boulders, and at site three was predominantly fine silt and mud.

A greater number of sessile species were recorded at the SGD than control sites in both seasons (8 vs. 5 in summer and 3 vs. 2 in winter). There was little cover of opportunistic green macroalgae at the SGD site in either season compared to the

~32 % coverage of *Ulva* spp. at site 2 and ~17 % coverage of *C. rupestris* at site 3. The barnacle *E. modestus* was found at site 1 in both seasons but was absent from both control sites (Table 3-2).

Table 3-3 Results of ANOSIM statistical test for percentage cover (% cover), macroalgal composition (species richness and biomass) and invertebrate composition (species richness and abundance), reporting *p*-values and R test statistic. The factor 'SGD' was tested for SGD sites and control sites within season (Summer (S1 x S2) and Winter (S1 x S3)), and the factor 'season' was tested for the SGD site between seasons (S1 (sum. X wint.)). For completeness, the two control sites were also compared statistically (Controls (S2 x S3)) *significant at $p < 0.05$.

Season (sites)	% cover	Macroalgal Composition	Macroinvertebrate composition
Summer (S1 x S2)			
R test statistic	0.96	0.944	0.336
p-value	0.008*	0.008*	0.008*
Winter (S1 x S3)			
R test statistic	0.848	0.392	0.876
p-value	0.008*	0.008*	0.008*
S1 (sum.x wint.)			
R test statistic	0.464	0.332	0.432
p-value	0.008*	0.02*	0.008*
Controls (S2 x S3)			
R test statistic	0.952	0.804	0.406
p-value	0.008*	0.008*	0.008*

As many of the *p*-values determined were constrained by the lowest limit imposed by the sampling design and the permutation based nature of derivation of the *p*-value (i.e. $p = 0.008$ (Anderson 2001)), more information about the statistical disparities may be determined by reference to the value of the associated R test statistic. The value of R is proportional to the magnitude of statistical disparity or difference. All comparisons of site 1 between seasons (S1 (sum. x wint.)) are associated with relatively low R values, as expected when comparing the same location between seasons. Comparison of the invertebrate composition (species richness and abundance) at the SGD and control site in summer (Summer (S1 x S2)), and the control sites (Controls (S2 x S3)) are associated with a relatively low R values, as is the comparison of macroalgal composition between the SGD and control site in winter (Winter (S1 x S3)). All other comparisons are associated with relatively high R values. The greater R values associated with the control sites' inter-season comparisons (Control (sum. x wint.)) than with the SGD site inter-season comparisons (S1 (sum. x wint.)) for percentage cover and macroalgal composition (in terms of species richness and biomass) are expected as the SGD site comparisons differ in season only (i.e. the same site sampled in different seasons), while the control sites comparisons differ in site location and season (i.e. two different sites samples sampled in different seasons).

The dissimilarity between the SGD and control sites is represented by good separation of samples from the SGD and control sites, and the low associated stress when plotted on nmMDS ordinations (Figure 3-3) for the summer data. Ordinations of both the winter data and comparing the SGD site between seasons gave similar patterns (see Appendix D).

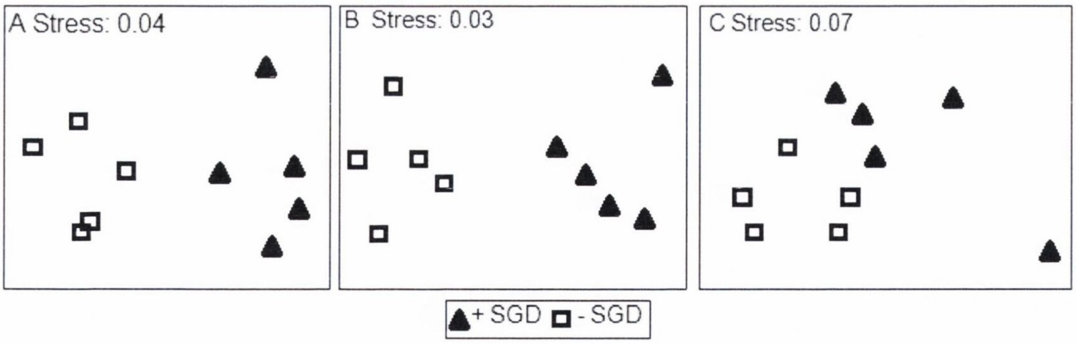


Figure 3-3 2D nmMDS ordinations of data from SGD site 1 and control site 2 showing (A) percentage cover data (√ transformed data), (B) algal species biomass data (√√ transformed data), and (C) macroinvertebrate species abundance data (√√ transformed data).

3.4.3. Algal community composition (biomass)

Table 3-4 Macroalgal mean and SE (1SE) biomass (g) per $0.15m^2$ data for SGD site 1 in summer and winter, and control site 2 in summer and 3 in winter. *n* = 5 in all cases. SE is not reported where only one sample was found for a site.

	Summer				Winter			
	Site 1		Site 2		Site 1		Site 3	
Species	**Mean**	**SE**	**Mean**	**SE**	**Mean**	**SE**	**Mean**	**SE**
Fucus ceranoides	58.65	11.60	11.25	2.74	128.37	16.83	17.87	9.84
Ulva spp.	0.01	0.01	2.33	0.78	1.42	0.92		
Polysiphonia spp.			0.02				0.29	0.25
Chaetomorpha linum	$3.80x10^{-4}$							
Cladophora rupestris							1.87	
Mean total	58.66	11.61	13.60	3.53	129.79	17.75	20.03	11.97

The SGD site and control sites differed in algal community composition as determined by the composition and biomass of harvested macroalgae ($p < 0.05$; Table 3-2). Thus, null hypothesis (1) of no difference in the community composition and biomass of harvested macroalgae at the SGD site and control sites can be rejected. The SGD site also differed in the composition and biomass of harvested macroalgae between seasons ($p < 0.05$; Table 3-2). This is represented by the low associated stress of the 2D nmMDS ordination of the underlying summer data matrix (Figure 3-3 (B); Stress: 0.03). Ordinations for the winter data and between season data showed similar patterns (see Appendix D). Macroalgal biomass was always dominated by *F. ceranoides* (Table 3-4), with more *F. ceranoides* and total algal biomass (~6 x) at SGD than control sites in both seasons ($p < 0.05$; Table 3-5). The *F. ceranoides* and total algal biomass found at site 1 in winter were over 2 x that found in summer ($p < 0.05$; Table 3-4; Table 3-5).

Table 3-5 Results of univariate Wilcoxon Rank sum comparisons of total macroalgal biomass, *F. ceranoides* biomass, and the abundances of the macroinvertebrate *J. albifrons*, reporting *p*-values and W test statistic. Summer (S1 x S2) and Winter (S1 x S3) indicate comparisons between the SGD site and control site within seasons. S1 (sum. x wint.) indicates comparison of SGD site 1 between seasons. For all, *n* = 5. *significant at *α* = 0.05.

	Biomass (g)		Abundance
Season (Sites)	**Total algae**	***F. ceranoides***	***J. albifrons***
Summer (S1 x S2)			
W test statistic	25	25	8.5
p-value	0.008*	0.008*	0.46
Winter (S1 x S3)			
W test statistic	25	25	25
p-value	0.008*	0.008*	0.009*
S1 (sum. X wint.)			
W test statistic	2	2	0
p-value	0.03*	0.03*	0.008*

3.4.4. Macroinvertebrate community composition (species abundance)

Table 3-6 Macroinvertebrate species abundance data (mean and SE) for SGD site 1 and control site 2 in summer, and SGD site 1 and control site 3 in winter. *n* = 5 in all cases. SE is not reported where only one sample was found at the site.

	Summer				**Winter**			
	Site 1		**Site 2**		**Site 1**		**Site 3**	
Species	**Mean**	**SE**	**Mean**	**SE**	**Mean**	**SE**	**Mean**	**SE**
Jaera albifrons	72.0	19.2	86.8	23.4	915.4	426.1	0.2	
Chaetogammarus marinus	7.2	3.3	15.8	2.9	16.4	8.4	0.4	
Ostracoda spp.			4.0	2.45				
Chironimid larvae			2.0	0.9			0.8	
Carcinus maenas			1.4	1.0			0.3	0.2
Halacaridae			0.4	0.2	0.2			
Enchytraeus albidus	1.2	0.7						
Beetle	0.2							
Procerodes littoralis					0.8	0.6		
Littorina littorea					2.2	1.6		
Corophium volutator							48.6	27.0
Tubifex costatus							5.4	4.9
Nereis diversicolor							1.8	0.7
Phyllodocidae							0.2	
Total # sp/site	4		6		5		8	

The SGD site differed from the control site in both seasons in terms of the abundance and composition of non-sessile macroinvertebrate species present (*p* < 0.05; Table 3-2). Thus, null hypothesis (3) of no difference in the structure and composition of the non-sessile macroinvertebrate assemblages at the SGD and control sites, can be rejected. The SGD site also differed between seasons in terms of the abundance and composition of non-sessile macroinvertebrate species present (*p* < 0.05; Table 3-2). These differences are visualised in the 2D nmMDS ordination of the underlying summer data matrix (Figure 3-3 (C); Stress: 0.07). Ordinations for the winter data and between season data gave similar patterns (see Appendix D). A greater total number of macroinvertebrate species were found at control than SGD site in both seasons (Table 3-6), though this was not statistically significant. *Jaera albifrons* (Leach, 1814) was the most abundant species at site 1 in both seasons, and site 2 (Table 3-6). This species was more abundant at the SGD site in winter than summer and at the SGD than control site in winter (Table 3-5). *J. albifrons* abundance was not significantly different between the SGD and control site in summer (Table 3-5), instead, differences in community composition were due to the relative abundances of other species present. In summer, four species were found at the SGD but not the control site and two species at the control site but not the SGD site in summer. All six species were present in relatively low abundances (mean 0.2 to 4 individuals). *Chaetogammarus marinus* (Leach, 1815) was the second most abundant species at site 1 in both seasons and site 2, while this species was nearly absent from site 3. Four macroinvertebrate species were recorded at control site 3 only; *Corophium volutator* (Pallas, 1766)*, Tubifex costatus* (Claparède, 1863)*, Nereis diversicolor* (O.F. Müller, 1776) and a member of the Phyllodocidae. One of these, *C. volutator, was* the most abundant macroinvertebrate species control site 3 (Table 3-6). As *C. volutator* was found at site 3 only, its abundance at site 3 was not compared statistically with any other site. *T. costatus* was present in low abundance, and *N. diversicolor* and the member of the Phyllodocidae were rare.

3.4.4.1. AMBI and M-AMBI

A small number of species were not included in the AMBI taxon list. In this case, I substituted for a closely related species, or I reduced the taxonomic level to the genus or family level where the higher level was present in the list. Where neither of these were possible, the species was ignored. The changes made are detailed in table 3-7.

Table 3-7 Changes made to enable inclusion in AMBI and M-AMBI calculations.

Original	Change
Coleoptera	Omitted
Ostracoda sp.	*Cypridina megalops*
Procerodes littoralis	Platyhelminthes (Order)
Phyllodocidae	*Phyllodoce* sp.

Table 3-8 Results of AMBI computation, showing the percentage of species belonging to each ecological group, the associated biotic index and the disturbance classification. %na indicates that no ecological grouping has been assigned.

	Site	%I	%II	%III	%IV	%V	%na	AMBI	BI	Disturbance Classification
Summer	S1	98.2	0	0	0	1.8	0.3	0.623	1	Undisturbed
	S2	93.3	3.6	3.1	0	0	0	0.124	0	Undisturbed
Winter	S1	99.7	0.3	0	0	0	0	0.01	0	Undisturbed
	S3	1	0.3	89.2	0	9.4	0	3.136	2	Slightly disturbed

The lowest AMBI was observed at the SGD site in winter and the highest AMBI at the winter 'relative control' site. For the SGD site in both seasons and the summer 'relative control' site, over 90% of species found belonged to ecological group I (%I above). Nearly 90% of species found at the winter 'relative control' site belonged to ecological group III. These differences were responsible for the SGD site in both seasons and the summer 'relative control' site being classes as 'undisturbed' while the winter 'relative control' site was 'slightly disturbed'. The lowest AMBI value was recorded at the SGD site rather than either of the control sites. The AMBI and biotic index (BI) indicate that the winter SGD site was the least disturbed site, followed by the summer 'relative control' site and the summer SGD site. The AMBI and biotic index indicate that the winter relative control had the worst ecological health of all four sites, when assessed in terms of benthic macroinvertebrates.

Table 3-9 Results of M-AMBI computation. Also shown are the AMBI, Shannon-Wiener (*H'*) and Richness (*S*) indices used to calculate the M-AMBI, and the associated ecological status. The 'Bad' and 'High' reference values for all four indices are indicated in the top two rows.

	Reference	AMBI	*H'*	*S*	M-AMBI	Status
	Bad	6	0	0	0	
	High	0.124	1.06	8	1	
Summer	S1	0.623	0.66	4	0.66	Good
	S2	0.124	1.06	6	0.91	High
Winter	S1	0.01	0.16	4	0.54	Good
	S3	3.136	0.9	8	0.80	High

The results of M-AMBI differ from those of AMBI. The results of the Shannon-Wiener index mirror that of the M-AMBI results. The Shannon-Wiener index, which is highly influenced by salinity, has a higher value at the control than SGD site in both years. The AMBI is less sensitive to salinity and displays the most ecologically favorable score for the SGD site in winter, followed by the control site in summer and the SGD site in summer. The control site in winter has the least favorable AMBI score. From M-AMBI, which is the most appropriate metric for high salinity gradients, the relative control sites were of ‘High’ ecological status, while the SGD site was of ‘Good’ ecological status in both years. The depreciation in status from high to good between the control sites is due to factors other than reduced salinity. The lowest AMBI was recorded at the SGD site in winter rather than at one of the control sites. It was expected that the lowest AMBI would be observed at one of the control sites and this would be used in the M-AMBI calculation as the ‘High’ reference value. The M-AMBI was calculated using both AMBI 0.01 and 0.124. Both AMBIs produced the same results and thus the relative control AMBI value (0.124) is cited as the ‘High’ reference condition in Table 3.9.

3.5. Discussion

SGD had decreased salinity, decreased pH, increased nitrogen content and altered temperature (higher in winter, lower in summer) compared to any marine compartment. While the temperature, salinity and pH of SGD remained constant between seasons, higher nitrogen concentrations were recorded in winter. Altered species percentage cover, macroalgal composition, and macroinvertebrate assemblage were also associated with SGD both between seasons and relative to the control site each season. Relative to the control sites, the SGD site (site 1) had greater total organism cover and *F. ceranoides* cover, but lower cover of opportunistic green macroalgae. Greater algal biomass (largely composed of *F. ceranoides*) was found at the SGD site when compared to both control sites. Greater macroinvertebrate abundance (largely composed of *J. albifrons*) was also found at site 1 relative to the winter control site. At the SGD site, macroalgal biomass (largely composed of *F. ceranoides*) and macroinvertebrate abundance (largely composed of *J. albifrons*) were higher in winter than summer. The results herein show that point source karst-channeled SGD alters the form and structure of the benthic intertidal food web in the receiving environment. This research found that point source karst-channeled intertidal SGD altered the composition and biomass of the macroalgal community - similar results were found by Migné et al. (2011) for diffuse SGD. In contrast to results found elsewhere for subtidal (Valiela et al. 1990) and diffuse intertidal SGD (Ouisse et al. (2011), opportunistic green macroalgae (*Ulva* spp. and *C. rupestris*) were prolific at control but not the SGD sites in the current study. This may indicate a difference in the effects of karst-channeled intertidal SGD relative to diffuse intertidal SGD and subtidal SGD and the influence of salinity rather than nitrogen additions in conditioning ecology in the current setting. The altered structure and composition of both the motile and sessile macroinvertebrate community documented in the current study corroborate the findings of previous work which documented changes in Polychaete assemblage associated with diffuse intertidal SGD (Miller and Ullman 2004; Dale and Miller 2008; Zipperle and Reise 2005). Similar to the results found here, SGD altered the composition, in terms of species richness, abundance and composition, of a wider section of the benthic intertidal food web, including gastropods, bivalves, decapods and amphipods, in Roscoff Arber Bay, France (Migné et al. 2011b). Contrary to the results documented herein however, Migné et al. (2011) found no effect of SGD on the total macroinvertebrate biomass, indicating a possible disparity between the effects of SGD on species diversity, abundances and biomass which may be particular to the effects of karst-channeled SGD. The Roscoff Arber Bay study found that SGD was associated with reduced species diversity (Migné et al. 2011) and dominance by a small number of species, often one single species (Ouisse et al. 2011). This is similar to the results herein where *J. albifrons* dominated the assemblage at both sites in summer and site 1 in winter, while *C. volutator* dominated the macroinvertebrate assemblage at site 3. As no previously published articles exist concerning the ecological effects of point source, karst-channeled intertidal SGD, this research updates the current knowledge on the ecological effects of this form of SGD.

Fucus ceranoides was the most prolific algae at all sites, present in greater cover and biomass at the SGD site relative to the control sites, and moreover, had greater biomass in winter than summer at the SGD site. Opportunistic green macroalgae (*Ulva* spp. and *C. rupestris*) were prolific at the control sites but not the SGD site. Although classically thought of as an estuarine species (Lein 1984), *F. ceranoides* tolerates rather than requires reduced salinity (Norton and South 1969), with germling development and growth at salinities between 8.5 to 34 psu, while detached tips of young algae can grow slowly at salinity 1 psu (Khfaji and Norton 1979). Reduced salinity is therefore not a requirement for settlement and development of the species, and prolific growth occurs in fully marine settings (Burrows 1964). Tolerance of reduced salinities however affords a competitive advantage over other less freshwater-tolerant macroalgal species. Numerous algal species are tolerant of depressed salinities, including a number of opportunistic green macroalgae (OGM) such as those found at the control sites. For example, most *Ulva* spp. proliferate at salinities between 20 – 30 psu, particularly in combination with elevated nitrogen concentrations (Kamer and Fong 2001a). The optimum salinity range for *U. intestinalis* growth on the other hand is 17 – 22 psu, and it experiences very low growth rates at salinities of less than 5 psu (Martins et al. 1999). Although one might expect to find reduced-salinity-tolerant OGM in areas influenced by nitrogen-rich freshwater SGD, this was not observed at the SGD site in Kinvara bay.

Traditionally, nutrient inputs are generally considered the primary factor affecting macroalgal production and growth rate. Recent studies, however, have found that salinity is the most important factor conditioning production and growth, followed by differences in dissolved inorganic carbon (DIC) and nutrient concentrations (Nygård and Dring 2008). In Kinvara, SGD was composed essentially of nitrogen-enriched freshwater. The deleterious effects of reduced salinity on growth can be offset by nutrient additions for both Fucoids (Nygård and Dring 2008) and *Ulva* spp. (Kamer and Fong 2001a). I suggest that profuse seepage at the SGD site (previously estimated to range from 5 - 30 m^3 s^{-1} (Drew 2008)) maintained freshwater conditions even at high tide, which allowed the development and proliferation of only the most freshwater tolerant species (i.e. *F. ceranoides*), in spite of additional nutrient availability. The ability to withstand consistently low salinities allowed the proliferation of *F. ceranoides* at the SGD site, and increased nitrogen availability resulted in increased algal biomass at the SGD compared to control sites.

Greater algal biomass was recorded in winter than summer at the SGD site, and at the SGD than control sites in both seasons. SGD water temperature, pH and salinity remained constant between seasons, but were altered compared to the receiving marine water. Groundwater temperature in Ireland generally ranges from 9.5 – 11.0 °C (Aldwell and Burdon 1986), congruent with the results of the current study except in summer when groundwater temperature was 16.4 °C despite SGD water being within the expected range, at ~11 °C. During operational monitoring of the same groundwater borehole, the Environmental Protection Agency (EPA) measured similar groundwater temperatures of 18 °C in June and 16.9 °C in September 2010 (Anthony Mannix, pers. comm. 2014). Groundwater temperature analysis of this borehole by the EPA from the years 2004 – 2012 found that the mean temperature was higher than reported average groundwater temperature, at 12.1°C, with a relatively high associated standard deviation (2.6 °C), and groundwater temperature ranged from 8 to 16.9°C (Mannix 2014). This borehole is located in a permanent pumping station which services the surrounding hinterland with potable water. The borehole is approximately 70 m deep and the water accessed via a permanent pump. The high readings recorded for this borehole in the current study and by the EPA relative to known groundwater temperature were likely an artifact of the pumping station mechanics. Irish Sea coastal temperatures can range from nearly 16 °C in summer to less than 8 °C in winter (Wilson and Rocha 2012); values congruent with those for marine samples in the current study. Summer SGD temperature was depressed and winter temperature elevated relative to the receiving marine water. Temperature influences the rate of metabolic reactions with increased temperature generally associated with increased reaction rates, and vice versa. This can be seen in non-nutrient limited estuaries where phytoplankton photosynthetic rates vary with season due to temperature, with higher rates in warmer seasons and lower rates in colder seasons (Eppley 1972). I suggest that nitrogen inputs combined with elevated temperature at the SGD site in winter promoted primary producer growth resulting in the observed increased algal biomass relative to summer when the temperature was similar but nutrient additions less. Similarly, nitrogen loading via SGD coupled with higher water temperature in winter may explain, at least in part, the increased SGD site algal biomass relative to the control. In summer, high nutrient concentrations, perhaps coupled with other abiotic factors resulted in greater algal biomass at the SGD than control site. Light availability for example is casual factor which differs between seasons. As there are longer light hours and a greater photon flux in summer than winter in Ireland, difference in light was not however considered as a factor which might explain the increased algal biomass in winter (when there are fewer day light hours and lower photon flux) than summer (when there are ~twice as many day light hours relative to winter and the photon flux is significantly greater).

The macroinvertebrate assemblage differed between the SGD and control sites and at the SGD site in different seasons. The differences between SGD and control sites were due mostly to differences in *J. albifrons*. *C. marinus* was the second most important species conditioning this difference in summer and *C. volutator* the second most important species in winter. The species list was similar at site 1 between seasons, but species abundances, mainly of *J. albifrons,* differed. The species list at the control sites between seasons was dissimilar, reflecting the difference in both site and season of sampling. Macroinvertebrates were more abundant at the SGD site than control site in winter, and at the SGD site in winter than in summer. These patterns reflect those of macroalgal primary production. The assemblages at all sites were primarily composed of herbivores, the biomass of which is essentially controlled by a combination of primary production and abiotic growth conditions.

The freshwater tolerant barnacle *Elminius modestus* on the other hand, was recorded at the SGD site but absent from the control site in both seasons. Intertidal barnacles are susceptible to changes salinity because of their fixed location in shallow costal waters (Bhatnagar and Crisp 1965). *Elminius modestus* regulates the opening of its opercular valves in response to external changes in salinity (Davenport 1976). The barnacle regulates its internal salinity to acclimatise to the external salinity and then resumes activity (Foster 1970). *Elminius modestus* displays activity, measured as cirral and valve movement, within the range 17 – 53 psu, with greatest activity at salinity ~33.5 psu (Davenport 1976, Foster 1970). *E. modestus* embryos can fully develop and hatch into functioning nauplii at salinities of 21.4-42.8 psu at 20 °C (Barnes and Barnes 1974). On release, *E. modestus* larvae can survive at salinities of 20 psu up to that of sea water (~35 psu) in a wide range of temperatures (~9°C -24°C) (Dassuncao, 2009). *E. modestus* can most likely still breed in salinities as low as 16 psu (Dassuncao, 2009). *E. modestus* has been recorded in areas of reduced salinity in a range of systems and is commonly found in brackish water in British estuaries (Jone and Crips 1954; Muxagata et al. 2004). *E. modestus* was introducted to Ireland from Australasia (Lawson et al. 2004). This species has been documented in Lough Hyne Marine Reserve, Ireland (Lawson et al. 2004). Lough Hyne is a fully marine reserve which receives freshwater inputs in places. *E. modestus* totally dominates other barnacle species at sites subject to freshwater influence in Lough Hyne (Lawson et al. 2004), comparable to the results found herein. Thus, *E. modestus* is tolerant of a range of salinities and may be an indicator of the presence of reduced salinity SGD as its ability to acclimatise to changing salinities affords this species a competitive advantage over competing, non freshwater tolerance sessile species. *E. modestus* may potentially be used as an indicator species for the presence of freshwater SGD in the Kinvara bay system.

Similar to the effects on primary producers, salinity, DIC concentration, pH and temperature will also control the form and structure of subsequent levels of the food web. Similarly to algae, temperature controls metabolic rate in higher organisms. Many invertebrates require specific physicochemical conditions to initiate progression between life cycle stages. Alterations to these conditions may modulate life cycle progression and subsequent development. Thus, alterations to physicochemical parameters (salinity, temperature and DIC concentration) may modulate the structure and composition of higher trophic levels beyond that expected from increased primary producer proliferation alone.

Previous work has documented the effects of diffuse intertidal, non-karst-channeled SGD on both the macroalgal and invertebrate community, with discharge altering Polychaete diversity and abundance (Miller and Ullman 2004; Zipperle and Reise 2005). Diffuse intertidal seepage has been associated with reduced species diversity (Migné et al. 2011) and dominance by a small number of species, often one single species (Ouisse et al. 2011). This is similar to the results herein where *J. albifrons* dominated the assemblage at both sites in summer and site 1 in winter, while *C. volutator* dominated the macroinvertebrate assemblage at site 3. The control sites differed in their macroinvertebrate assemblage. *J. albifrons, C. marinus, C. maenas* and Chironimid larvae were found at both sites, although in greater abundance at control site 2 than control site 3. Two individuals of the Halacaridae were found at site

2, while none were found at site 3. Four macroinvertebrate species found at control site 3 were absent from control site 2*; Corophium volutator, Tubifex costatus, Nereis diversicolor and a member of the Phyllodocidae.* This difference in macroinvertebrate assemblage was expected as comparisons of the control sites between seasons contain both inter-site and inter-season variability. Despite large differences in the terms of the identity of species, the R value associated with the ANOSIM test of macroinvertebrate composition at the control sites between seasons was relatively low; this reflects homogeny in richness and evenness parameters of species diversity between sites. The differences in macroinvertebrate assemblage between the two control sites likely reflects the effect of different sampling seasons (which also significantly altered the macroinvertebrate assemblage at site 1), and possibly also differences in abiotic factors associated with the different sites.

Jaera albifrons is an exclusively intertidal species, often found in estuaries, where it can occur in considerable numbers (Hayward et al. 1996, p.134) though it is rare in areas of very fast flowing water (Naylor and Haahtela 1966). Individuals of this euryhaline species have a high osmoregulation capacity, quickly acclimatise to salinity changes and occur in conditions of unstable salinity (Jones 1972). This species is thus well adapted to the both low and variable salinity at the seepage site, while additionally benefiting from shelter provided by macroalgae. The intertidal amphipod *C. marinus* is found on coarse sandy shores beneath stones or beached algae (Hayward et al. 1996, p.140). This euryhaline species can withstand reduced salinity but only for relatively short periods (Bolt 1983), explaining the decreased abundance of this species at the SGD site compared to the higher salinity control site 2 in summer.

Of the four macroinvertebrate species recorded at control site 3, *C. volutator* was the most abundant species, while the abundance of *T. costatus* was low, and the occurrence of the remaining two species was rare. *T. costatus* is a marine euryhaline species (Hayward et al. 1996, p.80) found in brackish waters, where it colonizes a variety of substrata (Brinkhurst 1964). *N. diveriscolor* tolerates a wide range of salinities (Beadle 1937) and is found in muddy sands, typically in sheltered inlets and estuaries (Hayward et al. 1996, p.84). *C. volutator,* an intertidal infaunal detritivore inhabits muddy shores and can be extremely abundant in estuarine mudflats (Meadows and Reid 1966). *C. volutator, T. costatus* and *N. diversicolor* all occur at reduced salinities, however, the occurrence of *N. diversicolor* and *C. volutator*, the most abundant species at site 3 is strongly conditioned by sediment type, with preference for muddy sand and silt sediment.

C. volutator will survive at salinities as low as 2.5 psu in a mud substrate, (increasing to 7.5 psu in the absence of mud), however breeding occurs only at salinities greater than 7.5 psu (McLusky 1968) and greatest growth rates are experienced at 15.4 psu (Mclusky 1967). Thus, *C. volutator* is characteristic of areas of reduced salinity, but not as low as that found at the SGD seepage site. Moreover, SGD at the observed rates (5 - 30 m^3 s^{-1} (Drew 2008)) can alter the local substrate by flushing away fine/light particles such as clay and silt. This is consistent with observations made during the current study: the substrate at the seepage site was mostly composed of pebbles, cobbles and boulders, in contrast with control site 3 where the substrate was comprised of fine silt and clay.

There exists a relationship between the velocity of water flow and sediment transport characteristics in terms of sediment deposition, transport and erosion. The Hjulström curve (Hjulström 1935) depicts this relationship, illustrating whether a current will erode, transport or deposit sediments based on water flow velocity and sediment grain size (Figure 3-5). The Hjulström graph may be used in conjunction with water velocity data to determine the sediment transport characteristics of SGD.

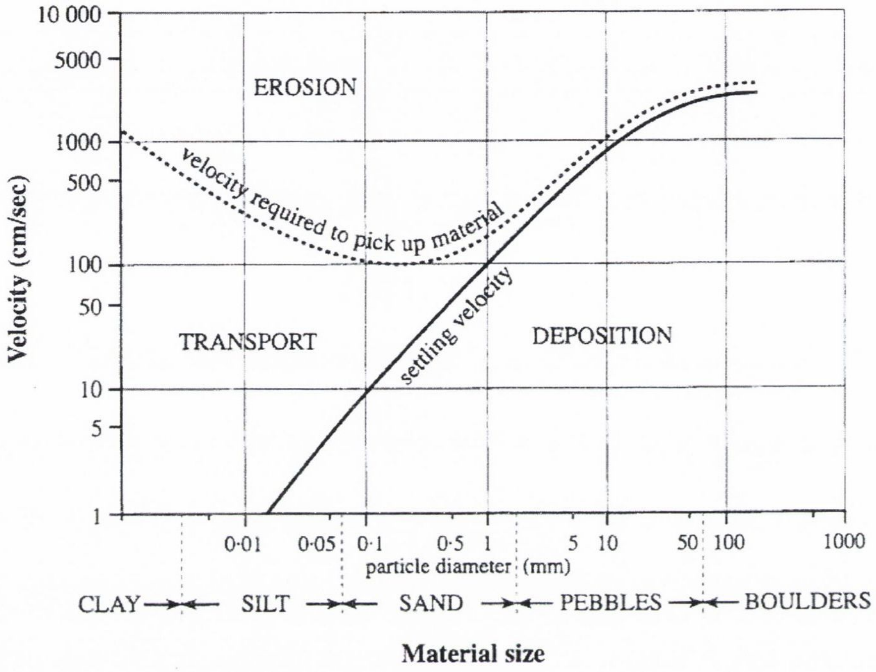


Figure 3-4 Hjulström curve depicting the relationship between water flow velocity (cm/sec) and sediment transport in terms of particle diameter (mm), adapted from Hampshire (2009, p.486). Both axes are on a log scale.

The velocity of SGD flow at the study site is not known, however the discharge rate/volumetric flow rate of SGD is 5 - 30 m^3 s^{-1} (Drew 2008). Discharge/flow rate and velocity are related metrics. The flow rate (*Q*) is the volume of fluid which passes by some location through an area during a period of time, and given by

$Q = V/t$

where *Q* is the flow rate, *V* is volume and *t* is time elapsed.

The average velocity of a river is given by

$$v_{av} = Q/A$$

where v_{av} is the average velocity, Q is the flow rate and A is the cross sectional area.

The cross sectional area of the SGD discharge site is not known, however it can be estimated in m^2 based on observations during field work carried out at the site. Using the estimated area and the known flow rate, SGD velocity may be calculated and the relationship between this velocity and sediment transport determined. From observation, the cross sectional area of the site of SGD discharge is estimated to be minimally, 2 m^2, and maximally, 4 m^2. This area data and the SGD flow rate (5 - 30 $m^3 s^{-1}$ (Drew 2008)) may be used to determine the average SGD velocity using the equation for average velocity (v_{av}) above. This velocity may be compared with the Hjulström curve (Hjulström 1935) to determine the sediment sizes and classes eroded at this velocity (Table 3-7).

3-10 Estimated SGD flow rate (5 – 30 $m^3 s^{-1}$ (Drew 2008)), estimated cross sectional area, calculated velocity, and the grain size diameter and size class of particles transported by associated water veloticy determined by reference to Hjulström curve (Hjulström 1935) (Figure 3-5).

			Particle transport	
Flux	Cross sectional area	Velocity	Grain size Diameter	Size class
5 $m^3 s^{-1}$	4 m^2	125 cms^{-1}	≤ ~0.1 mm	Clay and silt
5 $m^3 s^{-1}$	2 m^2	250 cms^{-1}	≤ ~2 mm	Sand and small pebbles
30 $m^3 s^{-1}$	4 m^2	750 cms^{-1}	≤ ~5 mm	Small pebbles
30 $m^3 s^{-1}$	2 m^2	1500 cms^{-1}	≤ ~20 mm	Large pebbles

Thus at the lowest estimated velocity based on largest cross sectional area and lowest water flow rate, the velocity of water flow at the SGD site would be sufficient to erode clay and silt. At the reduced cross sectional area, the flow rate of 5 m^3s^{-1} would also erode sand and small pebbles up to diameter ~2 mm. This increases to the erosion of large pebbles up to diameter ~20 mm at the highest estimated flux (30 m^3s^{-1}) and smallest estimated cross sectional surface area. Based on the most conservative cross sectional area estimate, SGD would have altered the sediment type by flushing away clay and silt particles at the lowest flow rate, increasing to small pebble transport at the highest flow rate. Therefore, the absence of *C. volutator* at the SGD site and its proliferation at the control site in winter may be due to differences in substrate composition as well as salinity.

The diversity and biotic indices inform on (a) the relevance of decreased salinity associated with SGD as a disturbance and (b) the relevance of parameters associated with SGD other than reduced salinity as a disturbance in the system. The Shannon-Wiener index, which is highly correlated with salinity (Zettler et al. 2007), was higher at the control than the SGD site in both seasons, by ~30 % in summer and ~80 % in winter. This illustrates the influence over benthic macroinvertebrate diversity associated with the disturbance caused by salinity. The AMBI, which is less sensitive to salinity, indicates that the SGD site in winter was the least disturbed site while the winter control site was the most disturbed site. The summer SGD and control site were undisturbed though the control site was slightly more disturbed than the SGD site. These results, combined with the increased dominance of a small number of species at the SGD site are consistent with non-salinity variables associated with SGD presenting a disturbance which occurs at low frequency (Sousa 1979a). In particular, the observed AMBI for the SGD site in winter supports this theory. The flux of SGD is higher in winter than summer due increased rainfall in winter. Therefore the presence of SGD is at its most stable and the frequency of occurrence of SGD disturbance, (where disturbance refers to the complete removal of SGD followed by presence of SGD) is at its lowest in winter. The elevated AMBI and biotic index as well as 'slightly disturbed' classification of the control site in winter may be due to sedimentation at this site in the absence of SGD.

The M-AMBI index, which is the most appropriate index in cases of large salinity gradients (Zettler et al. 2007) and which is derived from a combination of AMBI, Shannon-Wieners index and richness (Muxika et al. 2007) indicates that the SGD site is of 'good status' relative to the control sites which are of 'high status'. M-AMBI has been widely applied in Ireland (Atalah and Crowe 2010; Kennedy et al. 2011), Europe (Muxika et al. 2007) and the US (Borja and Tunberg 2011). It has been successfully applied across the oligohaline to euhaline salinity bands (Borja and Tunberg 2011). Here, the results of M-AMBI support the suggestion that non-salinity parameters associated with SGD present a disturbance to the system resulting in the 'good status' of SGD sites relative to the 'high status' of control sites. The M-AMBI result also supports the theory of SGD presenting a disturbance of low frequency as a disturbance of higher frequency would be associated with a decreased M-AMBI score and reduction in status.

Nitrogen concentrations were elevated at the SGD site compared to Galway bay and the within-bay control and control/marine sites. In summer, the nitrogen concentration of the control/marine was elevated relative to the control site. This was likely due to mixing of SGD and Kinvara bay water. The nitrogen concentration of the SGD/marine sample was comparable to that of SGD, possibly due to lack of dilution with sea water. In winter, SGD nitrogen content was ~8 to 10 times greater than that of the Galway bay water. A previous study found SGD nitrogen concentrations of ~230 µM in winter 2005 and autumn 2006 at this site (Cave and Henry 2011). This is significantly higher than those found in the current study. Kilroy and Coxon (2005) in a study of eight karst springs in the region found that P concentrations increased following the first autumn rains due to remobilization of soil P accumulated during the summer months due to application of fertilizer and manure. It is likely that nitrate behaves in a similar though less pronounced way (due to increased mobility through the aquifer compared to phosphate), possibly explaining the higher winter than summer SGD nitrogen concentrations in this study, and the elevated concentrations documented in autumn/winter by Cave and Henry (2011). Also, reductions in nitrate contamination from 2005/2006 to 2011/2012 might be explained by implementation of tighter groundwater pollution controls following the 2006 EC Groundwater Directive (Protection of Groundwater against Pollution and Deterioration Directive) (2006/118/EC). In Ireland, an EPA publication 'Water Quality in Ireland 2007 - 2009' reported a general decrease in groundwater nitrate contamination between the reporting period 2004/2006 to 2007/2009. Reductions in inorganic fertilizer application, improvements in storage of organic fertilizers and the implementation of land-spreading restrictions which may have reduced pressures, in addition to increased rainfall causing increased dilution of nitrate sources are cited as the probable causes for this decrease (Craig et al. 2010).

Sampling of the karst aquifer groundwater borehole ~1 km inland from the point of SGD discharge (from 2003 to 2010, sampled 2 - 4 times per season) and SGD from the current study site (from 2007 to 2010, sampled 3 - 4 times per season) were documented by the EPA in a recent national report 'Water Quality in Ireland 2007 – 2009' (EPA 2010). This report found that SGD samples always had higher nitrogen concentrations (136 ± 62 µM (O'Boyle et al. 2010)) than groundwater samples (78 ± 30.7 µM) Craig et al. 2010)), even when sampled on the same day. Also, the range of values for SGD water samples (9 to 257 µM (O'Boyle et al. 2010)) was greater than that for groundwater (6 to 136 µM (Craig et al. 2010)). These results are similar to those found in summer in the current study where the concentration of nitrogen in SGD was greater than that in adjacent groundwater. These consistent incongruities between the groundwater and SGD nitrogen concentrations highlight the complex nature of the aquifer and heterogeneity of the water contained within it. Further, these results indicate that there may be groundwater nitrogen additions and/or nitrification between the inland borehole and SGD site. This highlights the dynamic nature of karst aquifers, where nitrogen concentrations vary both seasonally and spatially and in non-systematic ways throughout the catchment.

The pH of marine water is typically ~8 and that of estuarine water 7.5 - 8.5 (Fogel et al. 1992). The pH of water from both Galway Bay and control site 2 was ~8, in line with expectations. The pH of the SGD was consistently ~7. The EPA measured the pH and alkalinity of SGD from the period 2007 to 2012, between 3 and 5 times per year. The average pH of the SGD over this period was 7.2, with standard deviation 0.26, and range from 6.8 to 7.9 (Anthony Mannix, pers. comm. 2014), consistent with the value recorded in the current study. The EPA found the alkalinity of SGD to range from 180 to 326 mg $CaCO_3$ with average 272.8 mg $CaCO_3$ and standard deviation 45 mg $CaCO_3$ (Anthony Mannix, pers. comm. 2014). The pH of a water body is determined by a number of factors, including concentrations and speciation of dissolved inorganic carbon. SGD can supply water column dissolved inorganic carbon (DIC) in coastal areas, and particularly carbonate coastal areas. Dorsett et al. (2011) estimated that 7 - 11 % of global coastal water DIC may be derived from SGD associated with karst and other carbonate systems. It is probable that SGD is a source of carbon loading at the SGD site in the current system. This loading of carbon as well as nitrogen at the SGD site may have promoted primary production and been responsible, at least in part, for the greater algal biomass at the SGD relative to control sites, particularly in winter when associated with the highest nitrogen loading.

Traditional intertidal rocky shore zonation was not observed at the study sites. Rocky shores are nearly universally characterised by the occurrence of specific organisms within distinct vertical bands. The upper and lower limits of the organisms' occurrence coincide with horizontal tidal levels, forming three broad bands/zones; (1) the supratidal zone, (2) the intertidal zone and (3) the subtidal zone. The supratidal zone, also called the splash zone is the zone located highest on the shore. It is only covered by water during storms and is moistened by the spray of breaking waves. The intertidal zone, also known as the littoral zone, is the zone between the highest and lowest limit of the tides. The subtidal zone, also known as the sublittoral zone, is the region below the intertidal zone which is largely permanently covered by water. Normally, in rocky intertidal systems, the intertidal zone is further subdivided into sequential strips or zones characterised by distinctive features and species (Stephenson and Stephenson 1949). Generally, there exist three zones, (1) the high intertidal zone, (2) the mid-littoral or mid-intertidal zone and (3) the lower littoral or low intertidal zone (Stephenson and Stephenson 1949). On the Irish and other Atlantic coasts, the high intertidal zone, is dominated by barnacles from genera such as *Balanus*, *Chthamalus* and *Tetraclita* (Stephenson and Stephenson 1949), lichens, encrusting algae (McNeill 2010) and snail species adapted to arid conditions and belonging to the genus *Littorina*

(Stephenson and Stephenson 1949). Barnacles give way to mussels and seaweed in the mid-littoral zone (McNeill 2010). The third zone, the lower littoral zone, is dominated by seaweeds (McNeill 2010). Within the intertidal zone, the upper limit for the occurrence of marine algae and sessile organisms is controlled by physiological limits on species tolerances' of physical stresses, mainly desiccation and heat (Connell 1972). Generally, invertebrate and algal species living higher in the rocky intertidal zone must tolerate longer periods of aerial exposure, and thus greater heat and desiccation stress than low intertidal species (Sanford 2002). Here, 'stress' refers to a reduction in the performance or fitness of an organism due to exposure to unfavorable environmental conditions (Menge and Sutherland 1987). Mechanical forces such as wave action and changes in factors like temperature and salinity may induce stress. The former (wave action) is a type of 'mechanical stress' and the latter two (salinity and temperature) are types of 'physiological stress' (Sanford 2002). The lower limit for the occurrence of marine algae and sessile organisms is often determined by the presence of predators or competing species (Connell 1972). The role of SGD in modulating the typical pattern of rocky intertidal zonation has not been considered. In the current system, the low and mid intertidal zones at the SGD seepage site (site 1) were covered with water, even at low spring tide, due to the profuse flux of freshwater SGD. The presence of SGD alleviated the desiccation and heat stress normally experienced in these two zones during low tide. Upon tidal inundation, the high intertidal zone at the SGD seepage site was submerged, however in fresh rather than marine water. Thus, the presence of freshwater SGD alleviated desiccation and heat stress but induced salinity stress for the marine organisms present. In rocky intertidal areas in receipt of freshwater SGD, the type of zonation present may be determined by salinity stress, rather than the typical heat and desiccation stress. The absence of visible rocky shore intertidal zonation at the control sites may be explained by the influence of bay water of decreased salinity (derived from SGD) at these sites upon tidal inundation.

The experimental design of the current study involved the same SGD site (site 1) but different control sites between season (site 2 and site 3). This design was used in order to have the maximum number of control sites (or, relative control sites) for comparison with the SGD site. Due to the nature of the system however (i.e. heterogeneous in substrate type at small/medium spatial scales), the substrate at site 1 and 2 were comparable, but distinct from that of site 3. As the SGD site and control site 3 were in close proximity (distanced < 100 m from each other), they were assumed to be still comparable as they are highly similar in all other abiotic factors. The difference in substrate type between site 1 and site 3 introduces a confounding factor, however, this difference was likely due, at least in part, to the presence/absence of SGD and thus may be considered an impact of the SGD, to be characterised in further investigations. The study herein highlights the difficulty associated with SGD ecological research in general and in particular in the current system. This presents somewhat of a caveat in the current study and requires that certain assumptions be made regarding the similarity between site 1 and site 3 despite the differences in substrate. The results of the current study might inform on a future superior sampling design.

A future experimental design might examine the ecological effects of SGD at a larger number of sites located at increasing distances from the point of discharge. This experimental design is however subject to another suite of restrictions. Kinvara village is located from ~600 to 800 m along the shoreline to the west of the SGD site. A surface-discharging sewage outflow pipe is located ~750 m from site 1, on the same shoreline. Kinvara village and the sewage outflow pipe severely restrict the placement of control sites along the western shoreline. Control sites may be located along the shoreline only at sufficient distances from the village and outflow pipe so as to ensure no impact of these sources of pollution on coastal ecology. At such distances from the SGD site there are however likely to be distance-dependent differences between any selected control site and the SGD site. While the substrate type differs from that of the SGD site to the east of the SGD discharge point, it is possible that the substrate becomes more similar to that at the SGD discharge site at increasing distances from the SGD site along the eastern coast. Again, however, at increasing distances, disparities in the ecological community may be present due to differences in environmental factors associated with location. An alternative design might focus on the area in the immediate vicinity of the SGD discharge point but with greatly intensified sampling effort, e.g. a transect of sampling stations (possibly 10-16 in total) separated by 2 to 10 m intervals to both the west and east of the SGD discharge point. Such a design would sample the benthic food web within the vicinity of SGD, but also at increasing distances, and would remain at a distance from the township of Kinvara and the sewage outflow pipe.

The salinity of the water covering the control sites at high tide was not measured in the current study. Given the relatively high flux of freshwater which discharges as SGD, it is likely that the salinity of water covering the control sites upon tidal inundation was lower than the open marine environment. Indeed, this was indicated in the results as all organisms sampled at all sites were estuarine species which had different levels of freshwater tolerance, with the most freshwater tolerant species recorded at site 1. As previously discussed, the control sites in the current study were 'relative control' sites which received minimal impact of freshwater the SGD, rather than absolute control sites which were devoid of any influence of SGD. Future sampling efforts should consider the salinity of the water column over the control sites at high tide, as well as that of the SGD and water in close proximity to the control sites at low tide in order to build a complete picture of the conditions to which both SGD and relative control site assemblages are exposed.

Differences in DIC concentration, nitrogen loading and water temperature are three postulated causes of the inter-season ecological differences at the SGD site as these parameters have been demonstrated to be ecologically relevant in other settings (Nygård and Dring 2008). There are, however, other SGD related variables which may differ between seasons. These may include, for example, water turbidity, organic matter content, and concentrations of pesticides, heavy metals and rare earth elements. There is a dearth of research on the ecological effects of intertidal SGD, with currently only five published studies (Dale and Miller 2008; Miller and Ullman 2004; Kotwicki et al. 2013; Migné et al. 2011a; Ouisse et al. 2011), none of which consider karst-channeled SGD. Thus, the primary aim of the current research was to determine the ecological alterations associated with karst-channeled intertidal SGD. A secondary aim was to begin to determine which causal agents of change associated with SGD which might be responsible for these ecological changes; in this case, nitrogen loading, temperature and DIC loading were considered. As the number of studies on the ecological alterations associated with SGD increases, there should also be an increase in the scope of such experiments to include characterisation of the causal agents of ecological change associated with SGD. Such future experiments might build on the results and conclusions contained in this and similar studies.

In conclusion, nitrogen-rich freshwater SGD was associated with increased algal biomass (which can, in some systems, be a symptom of eutrophication), altered algal community structure, and altered composition and structure of a second, higher trophic level (herbivore) by comparison to control sites. Site 1 in winter had the highest nitrogen concentration, macroalgal biomass and macroinvertebrate abundance, while all other measured physicochemical parameters of SGD remained the same between seasons, highlighting the relevance of nitrogen loading in conditioning SGD-associated ecology. The SGD site macroalgal and macroinvertebrate communities were adapted to variable and low salinities, while those found at the control sites were adapted to environments where the salinity was depressed relative to average seawater, but higher than that found at site 1. This illustrates the role of SGD-associated salinity in structuring the community at site 1 and informs about the cause of differences in assemblage between SGD sites and control sites. The temperature of SGD water was elevated relative to that of marine water in winter, possibly reinforcing conditions for increased productivity at the SGD site compared to the control site in winter. Altered carbon availability may also play a role in structuring the SGD associated community. Further experimentation is however needed to elucidate the relevance and mode of effect of DIC concentration and speciation over conditioning SGD-associated food webs.

Chapter 4. The ecological impacts of karst-channelled intertidal submarine groundwater discharge (SGD) on the rocky intertidal sessile community; Portugal, and a comparison with Ireland (Chapter 3)

4.1. Abstract

Freshwater submarine groundwater discharge (SGD) may deliver large nutrient fluxes to the marine environment, particularly nitrate, and particularly when karst-channeled. Though 25% of the world's coastline is karstic and SGD frequently occurs where karst aquifers are hydraulically connected to the sea, little work has been done to determine the ecological impacts of this form of SGD and its associated nutrient loads. This chapter reports on the ecological effects of karst-channeled intertidal SGD on the sessile assemblage inhabiting rock pools on an exposed section of south Portuguese coast. These findings are compared with those of a sister study of karst-channeled SGD in a contrasting ecoregion so that generalisations regarding SGD in general and karst-channeled SGD in particular, might emerge, specifically in terms of ecology. The current study was carried out on a rocky intertidal platform in the locale of Olhos de Água, in summer 2011. Two quadrat surveys were conducted in each of four SGD (salinity < 32 psu) and four relative control (salinity > 32 psu) rock pools. Salinity and nitrogen concentrations of 11 rock pools, three inland groundwater boreholes and the adjacent coastal marine environment were measured. Rock pool salinity was negatively correlated with nitrogen (as NO_3^- and NO_2^-) concentration ($r^2 = 0.979$; $p < 0.0001$). The concentration of nitrogen in the highest salinity rock pools was negligible, while the rock pool with the lowest salinity (4 psu) (and therefore greatest proportion of freshwater SGD) had the highest nitrogen concentration (~515 μM). Groundwater samples taken from the associated 55 km^2 aquifer, and located ~5 km^2 from the

intertidal plateau, had nitrogen concentrations which were variable (from ~258 to ~450 μM) and lower than that of the rock pool which had the greatest fraction of SGD. Quadrat surveys revealed that SGD had a significant effect on the structure of the sessile community. SGD was associated with altered percentage cover of sessile organisms ($p < 0.0001$), decreased organism cover (n.s.), and decreased species number (n.s). In the relative control rock pools, the fraction of freshwater SGD was negatively correlated with percentage cover of *Ellisolandia elongata* ($r^2 = 0.811$; $t = 4.88$; $df = 8$; $p = 0.002$), a species normally ubiquitous in similar systems devoid of SGD. This was the only significant relationship between the fraction of freshwater SGD and any ecological variable. Comparison of the results of the Irish and Portuguese studies revealed a number of commonalities, namely; (1) SGD consistently had depressed salinity and elevated nitrogen concentrations relative to marine compartments; (2) SGD was associated with altered sessile community structure; and (3) karst-channeled SGD consistently delivered nitrogen fluxes to the coastal marine environment, regardless of the intensity of catchment anthropogenic use and associated nitrogen contamination. Though SGD altered the structure and composition of the sessile community in both ecoregions, the form of these alterations were contrasting, highlight the system dependency of alterations.

4.1.1. Outline of Chapter 4 Experimental Design and Structure

Rock pools

1 2** 3 ** 4** 5** 6** 7 8 ** 9 10** 11**

SGD Rock pools Relative Control rock pools

Salinity (psu)

4.27>..>32.6>....................................36.7

(Rock pool 1) (Rock pool 6) (Rock pool 11)

Parameters measured:

- *Water chemistry analysis (all 11 rock pools):*

Dimensions; salinity; N concentration ($NO_2^-+NO_3^-$)

- ***Ecological survey (8 rock pools):*

Quadrat surveys (x2)

4.2. Introduction

Some 25% of the world's coastline is karst or carbonate in nature (Ford and Williams 2007). Coastal karst systems have a high propensity to transport large volumes of freshwater to the marine environment via submarine groundwater discharge (SGD) (Slomp and Van Cappellen 2004). Karst systems are generally characterised by pathways created by limestone dissolution which allow rapid infiltration and relatively rapid, unrestricted conduit flow of groundwater and associated dissolved and colloidal constituents (Coxon 2011). High transport rates, low retention times and generally well-oxygenated conditions result in reduced capacity for biogeochemical nitrogen removal reactions in karst aquifers (Slomp and Van Cappellen 2004). Thus, karst-fed SGD can deliver large fluxes of terrestrial pollutants, particularly nitrate, to the marine environment, rendering karst-channelled SGD a special case in terms of SGD.

Despite the documented potential for SGD to provide a pathway for terrestrial nutrients (particularly N) to the marine environment, the ubiquity of karst coastlines, and the high propensity of coastal karst systems to convey intertidal SGD, the ecological effects of karst-channelled intertidal SGD remain uncharacterised. Only two published studies concern the ecological impacts of karst-channeled SGD. Both consider SGD occurring subtidally (rather than intertidally), are from the Yucatan peninsula, Mexico (Mutchler et al. 2007; Carruthers et al. 2005), and consider the effect of a known pollutant (wastewater) conveyed via SGD on select seagrass species. Thus, there is a gap in the current state of the art of SGD associated ecology in terms of the ecological alterations associated with intertidal, karst-channeled SGD.

Intertidal rocky systems provide substrate for colonisation and succession/development of macroalgae (Sousa 1979b) and associated faunal assemblages. Macroalgae and rock crevices provide shelter and refuge for invertebrates (Coull and Wells 1983) and fish species providing important feeding, resting, spawning and nursery habitat, as well as other socioeconomically important ecosystem goods and services (Thompson et al. 2002). Despite their ubiquity along the coast and importance in terms of coastal ecosystem functioning, rocky shores are relatively understudied environments (Araújo et al. 2006; Atalah and Crowe 2010; D. Boaventura et al. 2002; Diana Boaventura et al. 2002; Bokn et al. 2003; Caceres-Martinez et al. 1993). Rock pools in particular provide useful units for studying the effects of processes due to their clearly delineated bounds, ease of accessibility in many cases, and by virtue of the fact that they present a number of experimental units in a relatively small geographic area, thus reducing the occurrence and effect of confounding variables.

SGD has been documented at the current study site, both intertidally and subtidally (Encarnacão et al. 2013; Carvalho et al. 2013). Encarnacão et al. (2013) found no significant effect of subtidal SGD on meiofauna; however the ecological implications of subtidal SGD are expected to differ from those of intertidal SGD due to differences in marine water dilution capacities in these two environments. Carvalho et al. (2013) described the presence of *Symsagittifera roscoffensis*, an acoel flatworm, in association with nitrogen-rich intertidal SGD at the sandy beach face directly adjacent to the karst plateau investigated in the current study. Though SGD has been documented in the study area, the effect of karst-channeled freshwater SGD on the ecology at the receiving intertidal karst environment has not been assessed.

The primary aims of this study were to assess if karst-channeled intertidal freshwater SGD was (a) associated with altered community structure and form of the sessile (non-motile/attached) assemblage and (b) determine if the alterations were associated with the fraction of SGD contained within rock pools. To address these aims, the following alternative hypotheses were proposed and associated null hypotheses tested:

(1)

Ha: The community structure and composition, assessed in terms of percentage cover attached species (i.e. macroalgae and sessile macroinvertebrates), will differ between the SGD rock pools and relative control rock pools.

Ho: There will be no difference in the community structure and composition, assessed in terms of percentage cover of attached species, between the SGD rock pools and relative control rock pools.

(2)

Ha: There will be a relationship/correlation between the fraction/proportion of freshwater SGD in rock pools and the observed ecological alterations.

Ho: There will be no correlation between observed ecological alterations and the fraction/proportion of freshwater SGD in rock pools.

A sister study was conducted in parallel with the current study, using similar methodologies but in a contrasting physical and climatic setting. Comparable systems were studied so that when considered together, generalisations about freshwater SGD in general, and karst-channeled SGD in particular, might emerge. These generalisations increase the knowledge and information content beyond that derived from studying each system individually, thus providing supplementary information without necessitating extra resource investment. In the discussion section of this chapter, the results of the study described herein are first discussed and then the sister study introduced and the results of both studies discussed in tandem. Thus, the third aim of the current chapter was to compare the results of the study presented herein with those of a sister Irish study in order to derive generalisations about SGD, and intertidal freshwater karst-channeled SGD in particular.

4.3. Methods

4.3.1. Study area

The study site is located in the Algarve region of southern Portugal, ~6.2 km east of the township of Albufeira, in the locale called Olhos de Água, meaning 'eyes of water' in recognition of the longtime presence of SGD-fed rock pools (Figure 4-1). The climate type is Mediterranean and semi-arid with little rainfall during hot dry summers, and winter rainfall infrequent but torrential when occurring (Bebianno 1995). Average annual temperature is 16.3 °C with a January minimum (9.9 °C) and August maximum (23.3 °C) (Stigter et al. 1998), and average annual precipitation is ~480 mm (Salles 2001). The temperature of the open coastal water ranges between 15 and 20 °C and salinity remains at ~35 psu. The regime is mesotidal with a maximum tidal range of 3.5 - 4 m during spring tide (Bettencourt 2004). Mainly Miocene carbonate forms the portion of the 'Lagos-Portimao' formation which sits at the edge of the karst limestone and dolomite Albufeira-Ribeira de Quarteira (ARQ) aquifer, and is the location of the karst intertidal sampling site. The ARQ aquifer underlies a 55 km^2 catchment area and has recharge of approximately 10 hm^3yr^{-1} (Stigter et al. 2009).

Groundwater in the region is subject to extraction pressure and nitrate contamination (Stigter et al. 2009). The growth of agriculture and tourism, the main industries in the region, has been associated with significantly increased water requirements in the past 30 years (Monteiro and Manuels 2004). Pre-1998, groundwater was the main source of water for all activities in the region (Stigter et al. 2009) despite increasing records of nitrate contamination in irrigated areas (Stigter et al. 2006) and coastal salt water intrusion (Carreira 1991; Stigter et al. 2009). Efforts to move away from groundwater extraction resulted in over 80 % of public water demands being met by surface water sources by 2002 (Stigter et al. 2009). Of the harvested water however, 69 % of demand goes to agriculture, 23 % to public water use and the remaining 8 % to other activities (golf courses, industry etc.) (Stigter et al. 2009). Irrigation water used in agriculture (~165 hm^3 yr^{-1}) is however primarily harvested from groundwater reserves (Stigter et al. 2009). Therefore groundwater is still subject to significant extraction. Fertilizer is the main source of diffuse nitrate groundwater contamination (Stigter et al. 2009). This contamination is exacerbated by irrigation with locally extracted, already nitrate-contaminated groundwater. Two aquifers in the region (the Campino de Faro and Luz-Tavira) have already been designated as nitrate vulnerable zones (NVZs) under the EU Nitrates Directive (91/676/EEC). The rate of coastal SGD at Olhos de Água has been estimated at 100 m^3 s^{-1} in total for the site (Almeida and Silva 1990). It is likely that coastal freshwater SGD will increase in the future as public water supply continues to move away from groundwater sources.

4.3.2. Study site

The study site, which is an outcropping intertidal karst plateau containing numerous rock pools of various dimensions, is devoid of visible vertical biological zonation. A number of rock pools are hydraulically connected to the karst aquifer and receive freshwater SGD at low tide via protrusions in the rock pool walls. Algae and mussels line the rock pool walls, and floors are overlain with a thin layer of debris ranging in size from sand and small pebbles to medium sized rocks. From consultation of a comprehensive survey of the species assemblages found at various shore heights on 27 Portuguese rocky shores, including Olhos de Água (Boaventura et al. 2002) it was determined that the ecology at the study site reflected that of the lower shore. This was due to the abundance of the mussel *Mytilus galloprovincialis* and the calcareous red algae, *Ellisolandia elongata*, both of which generally appear below mean tide level (MTL) on southern Portuguese coasts (Boaventura et al. 2002).

Rock pools have specific issues relating to variability in physicochemical parameters including pH, temperature, and other factors during emersion (Truchot and Duhamel-Jouve 1980; Morris and Taylor 1983). In the current study, the inter-rock pool variation in physicochemical properties associated with duration of emersion due is mitigated by the plateau nature of the sampling environment. The rock pools are located on a karst plateau which has negligible slope. Thus, the time interval between exposure of the lowest shore rock pools and highest shore rock pools is short, and rock pools are exposed to the atmosphere for approximately the same duration. The use of a plateau of rock pools is similar in effect to a 'blocked' experimental design where all experimental units (rock pools) are contained within the one block (the plateau) and treatments (i.e. the presence or absence of SGD) are randomly applied to experimental units (rock pools) within the block (plateau). It is thus assumed that the variability in physicochemical parameters is equal across all rock pools and does not cause inter-rock pool variation.

4.3.3. Experimental design

An ecological survey was conducted during low tide from May 2nd - 4th, 2011. The design of the ecological survey was treatment-control comparison, with replicates, using non-destructive point intersection quadrat surveys. To test null hypothesis (1) and determine the effect of SGD on organism percentage cover, two replicate quadrats were sampled in four SGD rock pools and four control rock pools. Control pools were identified using depressed salinity as a proxy for freshwater SGD input. Seawater salinity in the area is ~35 psu. As the rock pools were on a plateau, in some cases SGD rock pools overflowed into neighbouring rock pools which prior to the inflow did not have depressed salinity. To allow for this overflow effect which was deemed to be highly variable in time and flux (as opposed to the effect of immediate SGD infiltration), rock pools with salinities above 32 psu were considered relative control rock pools and those with salinities below were considered SGD rock pools. To test null hypothesis (2), rock pool salinities were used to determine the fraction of freshwater SGD present, and water samples were taken for determination of nitrogen concentration. Rock pool dimensions were also recorded, and from these total dimension (m^3) estimated. This data was used to determine if rock pool dimensions condition ecology. Furthermore, rock pool dimensions and the fraction of freshwater SGD in each pool (%) were used to estimate the volume of SGD in each pool and the total volume of freshwater SGD contained in the rock pools in the plateau at low tide. As water occupied 100% of the volume of each pool, rock pool dimension was used as a surrogate for total volume. The fraction of freshwater SGD present in each pool (%) was determined for the total volume of each pool, to provide an estimate of the total volume of freshwater SGD contained in each rock pool.

4.3.4. Water sampling and *in-situ* parameters

Water salinity data and other physicochemical parameters were determined and used in conjunction with the ecological quadrat survey data to address null hypothesis (2). Three inland groundwater boreholes (ARQ1 - ARQ3) (Figure 4-1 (A)), eleven rock pools (5 SGD and 6 control rock pools) (Figure 4-1 (B)), and the adjacent coastal marine environment were sampled. The water sample for nitrogen analysis of one of the SGD rock pools was lost. Nitrogen analysis was thus carried out on all three inland groundwater boreholes (ARQ1 - ARQ3) (Figure 4-1 (A)), ten rock pools (4 SGD and 6 control rock pools) (Figure 4-1 (B)), and the adjacent coastal marine environment. The groundwater borehole samples provided the groundwater end member and the coastal marine environment sample, the marine end member. Salinity measurements and water sampling of rock pools and the adjacent coastal marine environment took place during May 2nd - 4th, 2011 (Table 4-1). Aquifer water sampling took place on June 7th (ARQ1; 37° 05' 41.75" N, 008° 10' 57.21" W) and 16th (ARQ2; 37° 06' 11.30" N, 008° 12' 07.20" W, ARQ3; 37° 05' 47.20" N, 008° 12' 40.60" W). The reported rock pool salinity values are the lowest salinity measured in the pool, measured just prior to rock pool tidal inundation. Samples for nitrogen analysis were collected in 500 ml or 1000 ml acid-washed polyethylene (PE) bottles. One bottle was taken for each pool/site and from this 2 - 4 individual replicate water samples filtered. Bottles were thrice rinsed with sample water and samples collected from the fourth fill. Bottles were immersed closed and opened underwater to collect the sample. Water was immediately filtered through poly-ether-sulphone (PES) membranes (Rhizon SMS-10 cm; Eijkelkamp Agrisearch Equipment 0.1 µm pore size) into sterile 11 ml vacuum tubes (BH vacutainer) via a needle connected to the membrane with tubing. The first 5 ml of each sample was discarded as dead volume. Collection via this method precludes the need for further preservation (Luo et al. 2003; Seeberg-Elverfeldt et al. 2005). Vacuum tubes were stored at 4 °C until analysis. Water samples were analysed for combined NO_3^- and NO_2^- via flow injection analysis (FIA) on a Lachat™ QuickChem 8500 instrument following the dual determination of nitrite and nitrate via flow injection analysis using a cadmium column method (Anderson 1979; Johnson and Petty 1983). Salinity measurements were preformed in-situ using an YSI 600 (Yellow spring instruments) multiparameter probe, and GPS location recorded with a Garmin™ eTrex handheld GPS navigator.

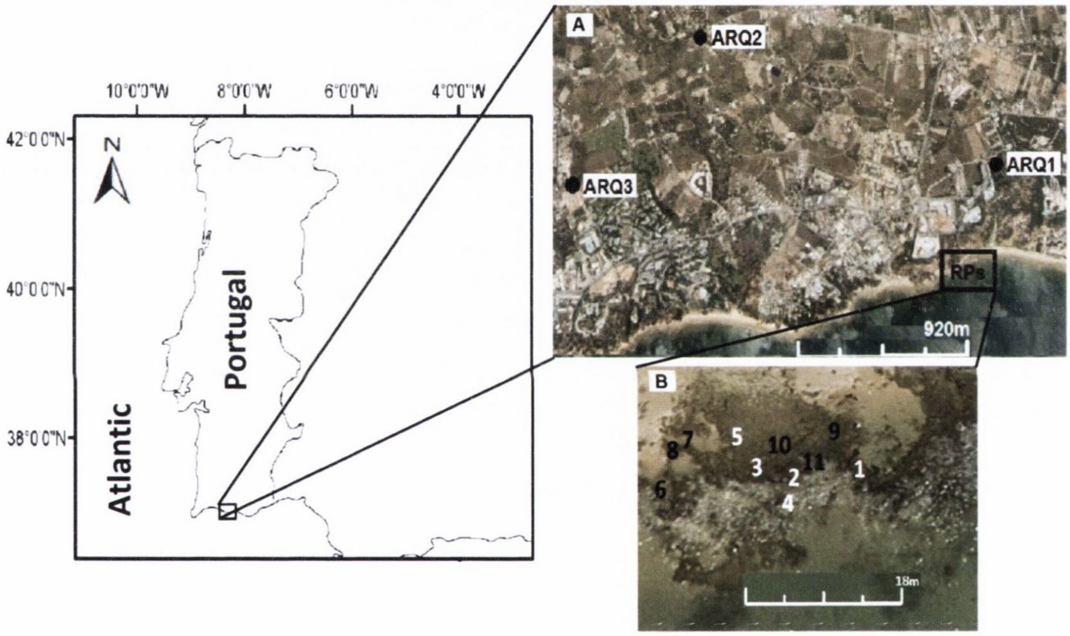


Figure 4-1 Location of groundwater borehole samples (ARQ1 – ARQ3) in the Albufeira-Ribeira de Quarteira (ARQ) aquifer and intertidal rock pool sampling platform (RPs) (A) (Google Earth 2007a), and locations of individual rock pools on karst platform, where white numbers (1-5) indicate the locations of SGD rock pools and black numbers (6-11) the locations of control rock pools (B) (Google Earth 2007b).

4.3.5. Quadrat survey

To test null hypothesis (1), quadrat surveys were conducted in four of the SGD rock pools (rock pools 2,3,4,5) and four of the control rock pools (rock pools 6,8,10,11). Two point intersection quadrat surveys were conducted per rock pool. Rock pools were roughly sketched and divided into ~25 cm sections. Sections were then numbered and random number tables used to determine quadrat locations. Quadrats were 24 cm^2 and sectioned at 2 cm intervals. A thin aluminum rod was inserted at each intersection and the intercepted substrate/species noted. Percentage cover data was calculated for species or substrate per quadrat as:

$$\frac{NIH_s}{\sum NIH} \times 100$$

where NIH_s is the number of intersection hits for the relevant species or substrate and ΣNIH the total number of intersection hits for that quadrat.

Quadrats were double strung to remove the compulsion to correct the path of the needle to account for perceived change in location/direction of the needle due to parallax error upon moving from air to water. Sampling was stratified, i.e. where one living species overlay another, all living species were recorded. Organism identification was done *in-situ* when possible, but where there was taxonomic uncertainty a sample was collected and identified in the laboratory.

4.3.6. Data analysis

The null hypotheses tested were (1) there is no difference in assemblage cover between the SGD and control rock pools and (2) there is no correlation between fraction of SGD and any ecological variables (cover of species or bare substrate). Data exploration is recommended prior to statistical analysis (Zuur et al. (2010)). The data exploration techniques suggested by Zuur et al. (2010) relate to (1) outliers in Y and X; (2) homogeneity in Y; (3) normality in Y; (4) trouble due to zeros in Y; (5) co-linearity in X; (6) relationships between X and Y; (7) interactions; and (8) independence of Y.

However, not all issues/techniques apply to all datasets (Zuur et al. 2010). Data exploration is separate from hypothesis testing, and what model to test should be made a priori based on biological understanding of the system (Burnham and Anderson 2002).

A number of steps were taken to address the requirements of data exploration set out by Zuur et al. (2010). Outliers and right-skewness are common in ecological data due to (1) the occurrence of rare species and (2) the tendency for abundances to bunch at smaller values with a long 'tail' of occasional larger counts (Clarke and Warwick 2001). A number of outliers and heterogeneity of variance were found in the current dataset. The dataset was transformed to remove the effect of outliers and stabilize the variance in the dataset (as per Zuur et al. (2010)). Due to the small sample sizes used it was not possible to test for normality; therefore non-parametric tests were used as these do not require normality of the dataset.

Correlation/co-linearity between X variables is not necessarily an issue in all studies. Co-linearity is only an issue in cases where the underlying question is which covariates are driving the response variables (Zuur et al. 2010, p.8). The underlying assumption of null hypothesis (2) is that some species will be negatively correlated with SGD (and thus positively correlated with each other), while other species will respond favorably to SGD and be positively correlated with SGD (and with each other). In the current study, the dataset is explored for co-linearity of variables, and this information retained for further use (to address null hypothesis (2)). Thus the structure of the current test/study removes the issues concerned with co-linearity.

Relationships between X and Y is also only an issue in some studies. In the current study, the analysis used to test null hypothesis (1) is based on the X variables only, while co-linearity between X and Y variables was the substance of null hypothesis (2). There wars a high degree of correlation between salinity and nitrogen concentration, and intuitively between these two variables and the fraction of freshwater SGD. Thus, three Y variables are reduced to only one variable (% fraction of SGD) for univariate statistical comparison with ecological variables. Observations from rock pools are assumed to be independent based on the sampling design with the clearly delineated structure of the rock pool units. Lack of interactions is assumed.

The raw multivariate dataset contains large numbers of zeros. Multivariate techniques are appropriate for multivariable ecological datasets containing a large number of joint zeros in the data as it is of relevance when two or more species are absent from sites (Zuur et al. 2010). The absence of two or more species may provide information about the ecological characteristics of a site, for example, that it contains conditions that are unfavourable to both species (Zuur et al. 2010). Sites which have the same joint absences may be ecologically more similar (Zuur et al. 2010). In the current study, the presence of joint zeros is significant. Specific statistical methods must be applied where there are a lot of zeros in the species dataset, depending on the relevance of the zeros. Specifically, where the sites are of interest (as in this case, SGD vs. controls sites), and there is an explanatory variable (as in this case, fraction of freshwater SGD), an ANOSIM test should be conducted (as per Zuur et al. (2007, p.21)). Thus, an ANOSIM (analysis of similarity, R test statistic) was conducted on a Bray-Curtis similarity matrix of square root transformed data to address null hypothesis (1) and test for a difference in composition of species cover due to SGD. A non-metric multi-dimensional scaling plot (nmMDS) was used to visualise differences in percentage cover associated with the presence/absence of the factor SGD (Zuur et al. 2007, p.261).

To address the second null hypothesis, it was necessary to determine the ecological variables associated with the greatest amount of systematic variation in the dataset and then determine if these variables are co-related with the fraction of freshwater SGD. Where the dataset contains not too many zeros, Principal Component Analysis (PCA), Correspondence Analysis (CA), Canonical Correspondence Analysis (CCA) or Redundancy Analysis (RA) may be applied to determine the systematic variation resulting from the species composition (Zuur et al. 2007, p.21). CCA and RA analyse both a set of response and a set of explanatory variables (Zuur et al. 2007, p.193). PCA and CA are used to analyse data without explanatory variables. PCA and CA assume there is no explanatory variable but instead seek any form of systematic variation in the data set. As the current requirement was to explore the response variables without explanatory variables, PCA or CA were suitable (Zuur et al. 2007, p.193). Whether CA or PCA should be applied depends on the form of the data and the relation between the ecological variables (Zuur et al. 2007, p.242). PCA is used in analyses where the relations between species responses are linear along the gradient, while CA is used to analyse where the species responses are unimodal along the gradient (Zuur et al. 2007, p.242). Based on the underlying assumptions of the hypothesis being tested (i.e. that there is a correlation between SGD and ecological variables), the species relations along the SGD environmental gradient were assumed to be linear. When plotted, the relations between variables along the gradient appear linear; however the variables only cover a short range of the gradient (i.e. restricted salinity range). As it was necessary to analyse species data which have linear relations along the gradient where the range of samples covers only a small part of the gradient, PCA was employed (as per Zuur et al. (2007, p.242)).

PCA was employed as an exploratory technique to determine the species responsible for the systematic variation in the dataset and provide information on the relationship between variables (Zuur et al. 2010). A commonly used application of PCA is to hypothesise that the most important components are correlated with some underlying variables. In this case, PCA was used to explore the data and determine which variables were associated with the greatest amount of variance in the data. It was hypothesised that these variables might co-relate with SGD, as an underlying variable.

The raw dataset contained a number of zeros. Two species being jointly absent contributes toward similarity in PCA and is informative. However, a large number of zeros in the dataset can have a strongly distorting effect on the analysis (Clarke and Warwick 2001). The inclusion of rare species has a distorting effect on PCA as it increases the number of zeros (Clarke and Warwick 2001). As it was desirable to gain information from joint absences without the analysis being distorted by the large number of zeros due to rare species, species recorded in only one pool were omitted from the PCA (as per Clarke and Warwick (2001), reducing the number of zeros in the dataset (as per Zuur et al. (2010, p.21)).

The fourth root transformed dataset was subject to Principal Component Analysis (PCA) to elucidate the main source(s) of systematic variation, producing a covariance based PCA (Clarke and Warwick 2001; Zuur et al. 2007, pp.200–202). PCA is a procedure for finding hypothetical variables (components) which account for as much of the variance in multidimensional data as possible (Davis 2011). Principal components (PCs) with eigenvalues greater than one (> 1) explain most variation in the data (Kaiser criterion (Kaiser 1960)) and were retained for further analysis. PC loadings (the correlation between a variable and the PC) measure the importance of each variable in accounting for the variability in the PC. PC variables with loading (correlation to the PC) > 0.6 were considered significant. A PC biplot with X-axis PC1 and Y-axis PC2 was constructed and relationships between variables interpreted. On a PC biplot, lines pointing in the same direction indicate that the corresponding variables are correlated with each other, with more acute angles between lines indicating a greater degree of correlation (Zuur et al. 2007, p.201). Lines pointing in opposite directions are negatively correlated with more obtuse angles between lines indicating a greater degree of negative correlation (Zuur et al. 2007, p.201). Lines with an angle of ~90 are uncorrelated (Zuur et al. 2007, p.201). The length of the line is proportional to the variance associated with that particular variable (in this case, species).

The aim of exploratory application of the PCA to the multivariate dataset is to reduce the complexity of the dataset so that univariate sources of the greatest amount of variation may be deduced. To address null hypothesis (2), PCA multivariate analysis was used to determine which species were associated with the greatest amount of variation in the dataset (as per Zuur et al. (2007, p.21)), and univariate linear regression was then used to determine if a relationship existed between the fraction of freshwater SGD and the variable(s) (species/bare substrate) associated with the greatest amount of variation in the dataset.

PCA was further used as an exploratory tool to determine if there was any effect of rock pool dimensions over the ecological variables (i.e. correlation between dimensions and ecological variables). As the data (dimensions and cover) were in different units (m^3 and %), data were normalised to make them comparable, and then the dataset fourth root transformed (Clarke and Warwick 2001).

Univariate regression analyses were used to assess the relationship between (a) salinity and nitrogen; and following PCA, (b) the fraction of SGD and ecological variables responsible for the greatest amount of variation in the dataset; and (c) the relationship between rock pool dimensions and the other variables. Multivariate analyses were conducted using PRIMER (Clarke and Gorley 2006) and regression analyses using SigmaPlot Version 12.2. Statistical significance was accepted if $p \leq 0.05$ ($\alpha = 0.05$) for all tests and the correlation coefficient (r^2) values are reported, where relevant, for significant relationships.

4.4. Results

4.4.1. Fraction of freshwater SGD, salinity and Nitrogen concentration

Table 4-1 Rock pools' GPS location (Lat/Long), salinity (psu), fraction of freshwater SGD (%), combined nitrate and nitrite concentration (NO_x, µM, *n* = 3 for all rock pools except rock pool 3 for which *n* = 2, ±1SD). Indicated are the length along the longest horizontal axis of each pool (Max. length); the maximum length along the axis perpendicular to this axis (Perp. length); and maximum depth (Max. depth); the total dimensions of each rock pool; and the volume of freshwater SGD in each rock pool. The total volume of freshwater SGD contained in all rock pools was 1.87 m^3.*Ecological survey. ** Data missing. ^{A}Measured data. ^{B}Calculated data.

Rockpool	/ Control	GPS location (lat(N)/long(W))	^{A}Salinity	^{B}Fraction (%) freshwater SGD	$^{A}NO_x$ µM (± 1SD, *n* = 3)	^{A}Max. length (cm)	^{A}Perp. length (cm)	^{A}Max. depth. (cm)	^{B}Dimension (m^3)	^{B}Vol. SGD (m^3)
1	SGD	37°5'23.56"N 008°11' 9.23"W	4.27	88.1	515.2 (± 0.6)	150	97	3	0.04	0.035
2*	SGD	37°5'23.54"N 008°11' 9.59"W	15.44	57.3	432.4 (± 2.8)	210	2	57	0.02	0.012
3*	SGD	37°5'23.57"N 008°11' 9.80"W	26.22	27.4	188 (± 0.1)	78	223	27	0.47	0.129
4*	SGD	37°5'23.47"N 008°11' 9.60"W	29.21	19	130.8 (± 1.2)	140	225	50	1.58	0.300
5*	SGD	37°5'23.70"N 008°11' 9.92"W	31.3	13.2	96 (±0.2)	530	230	27	3.29	0.434
6*	Control	37°5'23.47"N 008°11' 10.32"W	32.61	9.6	86.4 (±1.2)	71	148	38	0.4	0.038
7	Control	37°5'23.70"N 008°11' 10.24"W	34.18	5.2	37.1 (±0.1)	640	380	43	10.45	0.543
8*	Control	37°5'23.70"N 008°11' 10.28"W	34.53	4.4	**	540	240	42	5.44	0.239
9	Control	37°5'23.79"N 008°11' 9.39"W	34.6	4.1	33.7 (0.1)	238	206	68	3.33	0.137
10*	Control	37°5'23.63"N 008°11' 9.71"W	35.77	0.7	14.4 (± 0.1)	78	143	32	0.36	0.003
11*	Control	37°5'23.60"N 008°11' 9.51"W	36.07	0	15.3 (± 0.4)	372	173	73	4.70	0

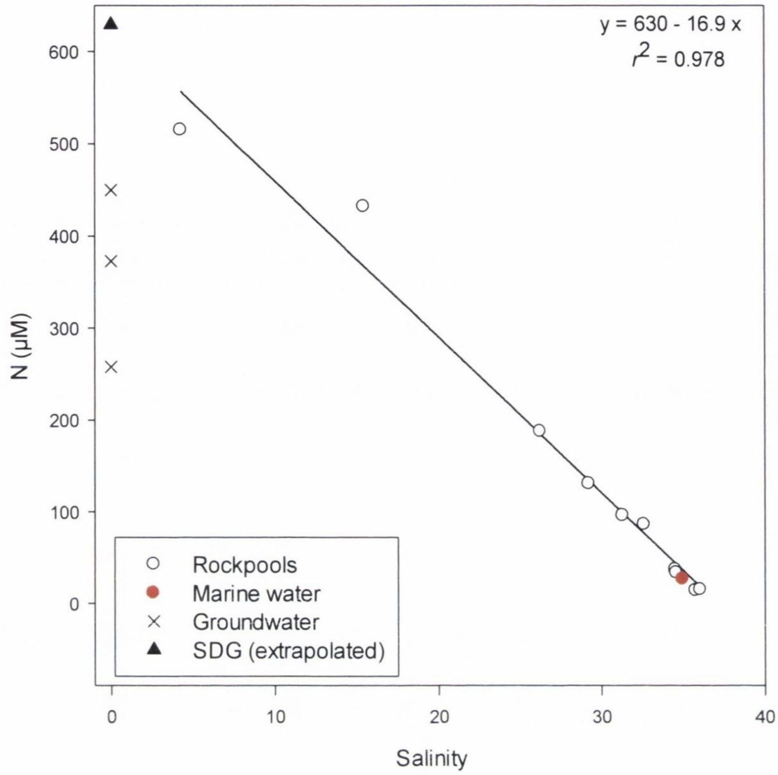


Figure 4-2 Linear regression model and correlation coefficients for rock pool nitrogen concentration (µM) and salinity (*n* = 10). Also indicated are the three groundwater samples (Groundwater), the coastal marine water end member (Marine water) and the extrapolated groundwater end member.

The highest rock pool nitrogen concentration recorded was ~515 µM at salinity ~4 (i.e. comprising 88% freshwater SGD), and the lowest concentration, ~15 µM, was recorded in two rock pools with salinity ~36 (~0% freshwater SGD) (Figure 4-2). The nitrogen concentration of coastal marine water, i.e. the marine end member (~27 µM) was greater than that of the two fully marine control rock pools (~15 and ~14 µM). Groundwater nitrogen concentrations were 450 ± 18 µM (*n* = 3), 313 ± 13 µM (*n* = 4) and 258 ± 8 µM (*n* = 3) at ARQ1, ARQ2 and ARQ3, respectively. Linear regression analysis identified a significant negative correlation between salinity (used as a proxy for freshwater SGD) and rock pool nitrogen concentration ($r^2 = 0.979$; t = -24.21; *df* = 11; $p < 0.0001$, where H_o: slope = 0) (Figure 4-2). The value of the intercept indicates the extrapolated nitrogen concentration of fresh SGD without any marine fraction (i.e. the extrapolated groundwater end member) at 629 µM with 95% confidence interval 573 to 685 µM (n = 8, t = 2.3). The extrapolated groundwater end member is elevated relative to the measured inland groundwater concentration.

The data points for the two highest nitrogen concentration (lowest salinity) might suggest a potentially curved relationship between nitrogen concentration and salinity from this point. This, however, may be an artifact of the relatively small number of data points as there are only two data points between the salinity range 0 and 25 psu. For example, if fewer data points had been obtained, say terminating at the salinity 29, nitrogen concentration 130.8 µM, the last two data points in dataset would also indicate a curved relationship from this point. Plotting a curved relationship using this restricted dataset guided by the curved nature of the last two points would produce a graph which is very distinct from that obtained from plotting the complete dataset – either curved or linearly. The curved nature of the last two data points may be due to the small number of data points within the range rather than a true reflection the relationship between salinity and nitrogen concentration. Thus, the data were plotted as a straight line (guided by the relationship of the data between 25 and 37 salinity where the majority of the data points lie) using the two data points at the lowest salinity as indicative guides rather than plotting a curved line which would give these points a large amount of leverage over the form of the plot.

Based on the fraction of freshwater SGD in each rock pool and estimated rock pool dimensions (volume), the total volume of freshwater SGD entering the rock pools on the karst plateau is estimated at 1.87 m^3 over a two hour emersion period, or 3.74 $m^3 day^{-1}$. This equates to a discharge rate of 0.015 m^3 min^{-1}. This is likely a conservative estimate as SGD may discharge from sites on the plateau which are not the sites of rock pools, and some SGD rock pools with relatively low SGD influx may have been omitted in the sampling campaign. Nonetheless, this discharge rate provides a useful approximation.

4.4.2. Community composition (percentage cover of sessile (non-motile/attached) species)

Table 4-2 Rockpool percentage cover data. Values represent the average of two quadrats sampled in each rock pool. All values reported are mean ± SD per rock pool. Also given are details of the fraction of freshwater SGD, rock pool salinity (psu) and combined nitrate and nitrite concentration (NO_x, µM, $n = 3$, ±1SD). The substrate was carbonate rock overlain with a thin layer of debris ranging in size from sand and small pebbles to medium sized rocks. ** indicates data missing

	SGD				Control			
Rockpool	**2**	**3**	**4**	**5**	**6**	**8**	**10**	**11**
Fraction fresh SGD (%)	**57.3**	**27.4**	**19.0**	**13.2**	**9.6**	**4.4**	**0.7**	**0**
Salinity	**15.4**	**26.2**	**29.2**	**31.3**	**32.6**	**34.5**	**35.7**	**36.1**
NO_x (µM ± 1SD, $n = 3$)	**432.4 (± 2.8)**	**188.0 (± 0.1)**	**130.8 (± 1.2)**	**96.0 (±0.2)**	**86.4 (±1.2)**	**	**14.4 (± 0.1)**	**15.3 (± 0.4)**
Substrate	55.6 ±7.5%	45.9 ±15.6%	55.8 ±0%	78.2 ±18%	45.8 ±4%	59.7 ±3%	18.3 ±0.4%	26.4 ±26.5%
Ulva spp.		29.8 ±19%	6.3 ±1.8%	0.7 ±1.0%	6.5 ±7.2%	7.4 ±7.6%	15.5 ±4.4%	4.9 ±2%
Mytilus galloprovincialis	29.1 ±5.2%	2.1 ± 3%	3.1 ±4.4%		7.4 ±8.5%		7.5 ±10.6%	
Ellisolandia elongata					9.2 ±10.6%	24.5 ±2.9%	51.6 ±22.7%	51 ±0.5%
Gelidium spinosum		22.2 ± 0.3%	0.3 ±0.5%		31.1 ±1.2%		7.0 ±8.0%	
Gigartina acicularis	15.0 ±2.8%			21.1 ±17%				
Chordaria flagelliformis			34.4 ±6.8%					
Cystoclonium perpureum								17.7 ±25.0%
Phaeophyceae						8.1 ±7.2%		
Dictyota dichtoma						0.3 ±0.5%		
Plocamium cartilagineum	0.4 ±0.5%							

Species from ten different genera were recorded; nine macroalgae, and one bivalve mussel, *Mytilus galloprovincialis* (Linnaeus, 1978). Green algae of the genus *Ulva* (*U. intestinalis* (Linnaeus, 1953) and *U. rigida* (Agardh, 1823)) were the most abundant, followed by the calcareous red algae *Ellisolandia elongata* (Agardh, 1852), and members of the Rhodophyceae; *Gelidium spinosum* (Hauck, 1883), *Gigartina acicularis* (Lamouroux, 1813), *Chordaria flagelliformis* (Agardh, 1817) and *Cystoclonium purpureum* ((Hudson) Batters, 1902) (Table 4-2). There were relatively minor contributions from three other algae, two of which were red algae, *Plocamium cartilagineum* ((Linnaeus) Dixon, 1967) and *Dictyota dichotoma* (Lamouroux, 1809), and one unidentifiable member of the Phaeophyceae. *E. elongata* was found only in control rock pools. On average, more species were recorded in control than SGD rock pools (3.75 ± 0.25 vs. 3.25 ± 0.48, $n = 4$), and, generally, greater bare substrate was associated with SGD (58.87%) than control (37.55%) rock pools (non significant). SGD and control pools differed based on the percentage cover of sessile organisms (ANOSIM; R = 0.443; $p < 0.001$, $n = 8$), thus null hypothesis (1) of no difference in the community structure and composition between SGD and control rock pools, assessed in terms of percentage cover of sessile (non-motile/attached) species, is rejected. This difference between SGD and control rock pools is reflected in good spatial separation of SGD and control pools and low associated stress when plotted on a 3D nmMDS ordination (Figure 4-4; Stress: 0.06). The two quadrats sampled from SGD rock pool 3 and one quadrat sampled from control rock pool 6 are located in close proximity on the 3D nmMDS plot (grouped by a circle in Figure 4-4). The coverage in the relevant quadrat from rock pool 6 was ~43 % bare substrate, ~30 % *G. spinosum*, ~11 % *Ulva* spp., ~2 % *E. elongata* and ~13 % *M. galloprovincialis.* Rockpool 4 quadrats and the control rock pool 6 quadrat show a high degree of commonality in the species present, with all species found in rock pool 3 also occurring in the rock pool 6 quadrat. The quadrat from rock pool 6 contains only one species which is not found in the control rock pool, *C. mediterrranea*. The percentage bare substrate recorded in the SGD rock pool 3 quadrats (45.9 ± 15.6, mean ± 1SD) and the rock pool 6 quadrat are the most similar bare substrate values recorded across the rock pools, as are the values for *G. spinosum.* The close grouping of the quadrat from control rock pool 6 with those from SGD rock pool 4 is based on similarity in both the species present and their coverage. The separation of the quadrat in rock pool 6 from those of rock pool 3 is due largely to the presence of *E. elongata* in the control rock pool and its absence in the SGD rock pool, along with difference in cover of other species, particularly *M. galloprovincialis* and *Ulva* spp.

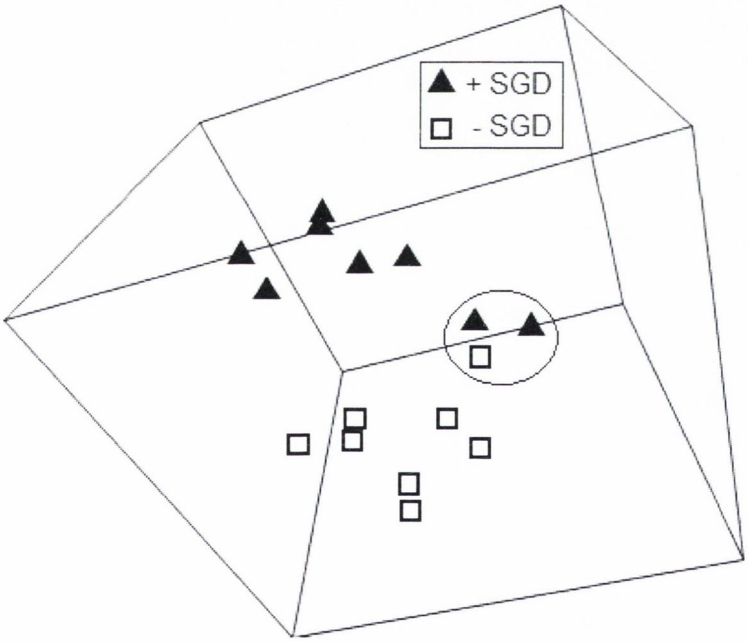


Figure 4-3 3D nmMDS ordination of underlying Bray-Curtis constructed similarity matrix of rock pool percentage cover data (√ transformed data, *n* = 4 rock pools with 2 quadrats in each rock pool). Stress: 0.06. Three quadrats, two from SGD rock pool 3 and one from control rock pool 6, are located in close proximity to the each other and indicated by an encompassing circle.

4.4.3. Correlation between fraction of freshwater SGD and ecological variables

4.4.3.1. PCA results

Table 4-3 Results of the Principle Component Analysis (PCA) carried out on fourth root transformed data showing eigenvalues, and individual and cumulative (%) contribution to variability in each principal component. Eigenvalues > 1 are in bold.

Principal Components	Eigenvalue	Individual (%)	Cumulative (%)
1	**2.61**	**43.6**	**43.6**
2	**1.94**	**32.5**	**75.1**
3	8.16×10^{-1}	13.6	89.7
4	3.48×10^{-1}	5.8	95.5
5	1.99×10^{-1}	3.3	98.9
6	6.85×10^{-2}	1.1	100

Table 4-4 PC loadings obtained from PCA. Loadings above the critical threshold value of 0.6 are indicated in bold.

Variable	Eigenvector coefficients/loadings	
	PC1	PC2
Substrate	0.157	0.011
Gigartina acicularis	0.519	0.198
Gelidium spinosum	-0.216	**-0.723**
Ulva spp.	-0.152	-0.277
Ellisolandia elongata	**-0.764**	0.230
Mytilus galloprovincialis	0.229	-0.555

PCA-generated eigenvalues are shown in Table 4-3. The first two principal components (PCs) have eigenvalues greater than one. PC1 is associated with 43.3% of the variation in the data, and PC2 with 32.4%. Loading factors associated with PC1 and PC2 are shown in Table 4-4, where significant loading factors (> 0.6) are highlighted in bold. *E. elongata* is highly negatively correlated (-0.764) with PC1, and *G. spinosum* with PC 2 (-0.723). High correlation between PC1 and a variable indicates that that variable is associated (positively or negatively, contingent on the sign of the loading) with the direction of maximum amount of variation in the dataset.

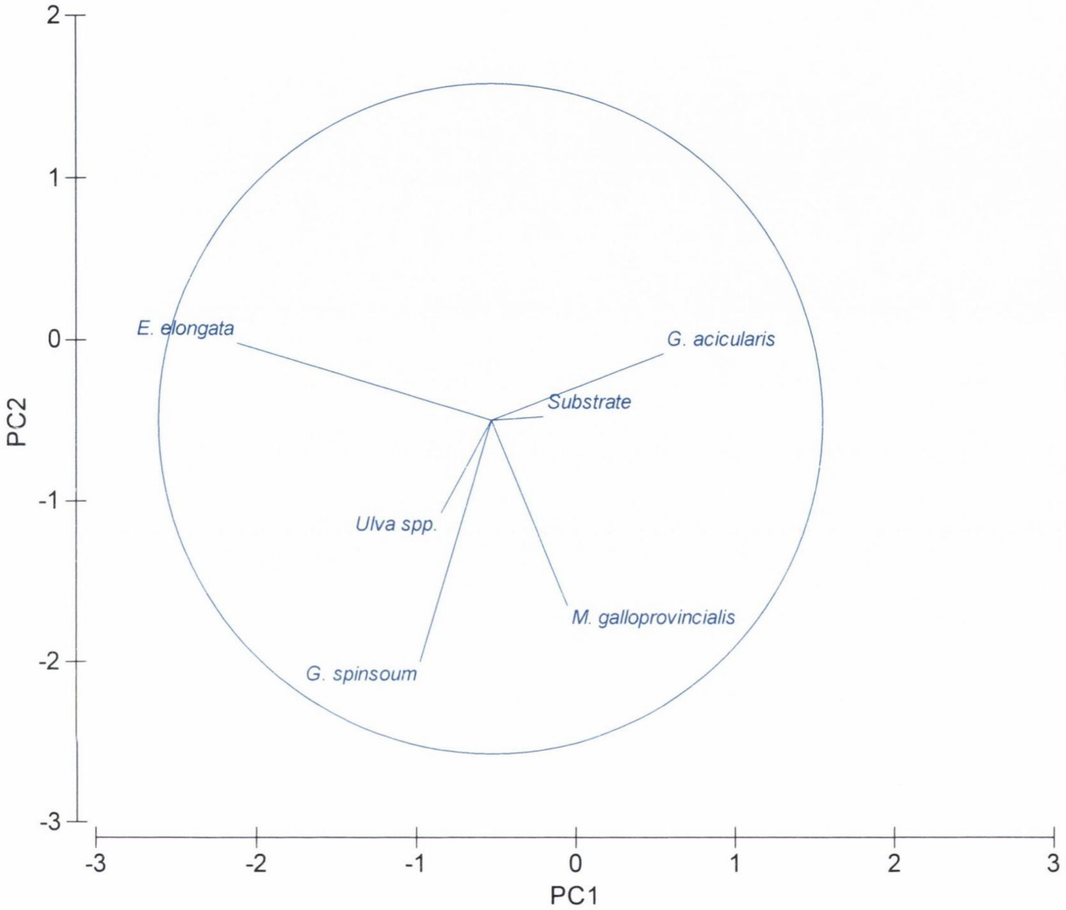


Figure 4-4 PC biplot, with x-axis PC1 and y-axis PC2 based on table 4-4 above.

The high degree of negative correlation between PC 1 and *E. elongata* is evident in the PC biplot (Figure 4-5). Also, this is the longest line in the plot, congruent with it accounting for the greatest amount of variation in the dataset. *G. spinosum* is highly negatively correlated with PC2 in the biplot, as expected from the output in table 4-4. From the PC biplot, it appears that there may be two or possibly three underlying explanatory variables causing variation in the dataset, one associated with *E. elongata*, one associated with the group containing *G. spinosum*, *Ulva* spp. and *M.*

galloprovincialis, and a third possibly associated with the group containing *G. acicularis* and substrate.

The angle between the *E. elongata* line and *G. spinosum* line is ~90° indicating that these are not correlated. Thus, the variation in *E. elongata* and that in *G. spinosum* are associated with different variables. The angle between *G. spinosum* and *Ulva* spp., and *G. spinosum* and *M. galloprovincialis* are acute, indicating positive correlation between these three variables. Thus, at least some amount of the variation in these three variables is associated with the same one factor. *G. acicularis* and substrate are at acute angles to each other, indicating a high degree of correlation between these variables. The *G. acicularis*/substrate group is at a right angle to the *G. spinosum/Ulva* spp./*M. galloprovincialis* group, and pointing in the opposite direction to *E. elongata*. Thus, the *G. acicularis/*substrate group is largely uncorrelated with the *G. spinosum/Ulva* spp./*M. galloprovincialis* group and negatively correlated with *E. elongata*. The variable which is associated with the response in *E. elongata* is likely also responsible for the observed pattern in bare substrate and *G. acicularis,* or the response observed in the *G. acicularis*/substrate group may be due to the presence of a third explanatory variable.

4.4.3.2. Regression of significant variables and SGD

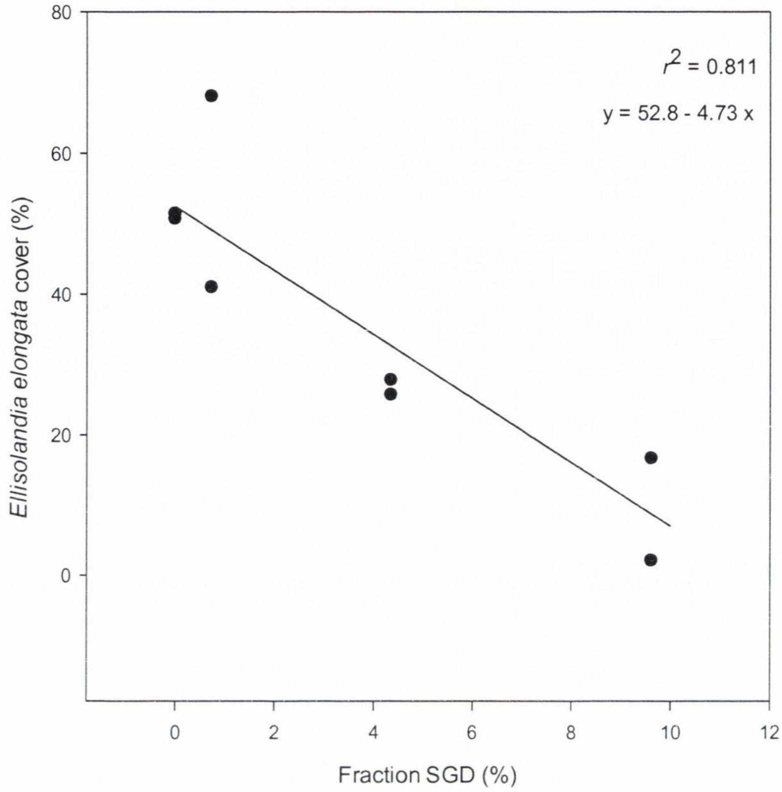


Figure 4-5 Linear regression model and correlation coefficients of the fraction (%) of freshwater SGD and *Ellisolandia elongata* cover (%) in two replicate quadrats in each of the four control rock pools.

Following interpretation of the PCA output, linear regression was used to determine if there was a relationship between the fraction of freshwater SGD and each of the variables *E. elongata*, *G. latofolium* and *G. acicularis*. *G. spinosum* and *G. acicularis* were selected as they had the longest line in each of their groupings (the *G. spinosum/Ulva* spp./*M. galloprovincialis* group and *G. acicularis*/substrate, respectively) and thus were associated with the largest amount of variation in their groups. A significant negative relationship was found between *E. elongata* cover and fraction of SGD in the relative control rock pools (Figure 4-7; r^2 = 0.811; t = 4.88; *df* = 8; *p* = 0.002, H_o: slope = 0). This was the only significant correlation. Thus, null hypothesis (2) (no correlation between observed ecological variables and the fraction/proportion of freshwater SGD) is rejected.

The negative correlation between *E. elongata* and the substrate/*G. acicularis* group in the PC biplot suggests a positive correlation between fraction of freshwater SGD and both bare substrate and *G. acicularis* cover. While there was, on average, more bare substrate in SGD rock pools than control rock pools, there was no significant correlation between SGD and substrate when compared using linear regression. From inspection of results, *G. acicularis* was found in only two rock pools, both of which were in the SGD category, thus not providing sufficient data for true interpretation. This reduces the number of explanatory variables underling the dataset to two (1) that associated with variation in *E. elongata* (i.e. SGD, at least in part) and (2) a second variable, uncorrelated with the fraction of freshwater SGD and associated with the systematic variation in *G. spinosum*, *M. galloprovincialis* and to a far lesser extent, *Ulva* spp. The variation associated with the latter variable is that associated with PC 2, and accounts for ~32 % of the variation in the dataset. *G. spinosum* was highly correlated (loading > 0.6) with PC2, while the correlations between this variable and *M. galloprovincialis* and *Ulva* spp. were relatively low (< 0.6). Thus, this variable is associated with a relatively large/significant amount of the variation in *G. spinosum*, but less or an insignificant amount of variation for both *M. galloprovincialis* and *Ulva* spp.

4.4.3.3. Rockpool dimensions and ecological variables

Table 4-5 Results of the Principle Component Analysis (PCA) carried out on normalised, fourth root transformed data showing eigenvalues, and individual and cumulative (%) contribution to variability in each principal component. Eigenvalues > 1 are in bold.

Principal Components	Eigenvalue	Individual (%)	Cumulative (%)
1	**2.56**	**36.6**	**36.6**
2	**2.39**	**34.1**	**70.7**
3	8.63×10^{-1}	12.3	83.0
4	5.13×10^{-1}	7.3	90.3
5	3.64×10^{-1}	5.2	95.5
6	1.79×10^{-1}	2.6	98.1
7	1.33×10^{-1}	1.9	100

Table 4-6 PC loadings obtained from PCA. The critical threshold value was set at 0.6.

Variable	Eigenvector coefficients/loadings	
	PC1	PC2
Dimensions	0.041	-0.593
Substrate	0.457	0.115
Gigartina acicularis	0.523	0.158
Gelidium spinosum	-0.378	0.342
Ulva spp.	-0.464	0.240
Ellisolandia elongata	-0.298	-0.338
Mytilus galloprovincialis	0.008	0.568

Principal Component Analysis (PCA)-generated eigenvalues for the dataset including the variable 'dimension' are shown in Table 4-5. The first two principal components (PCs) have eigenvalues greater than one. PC1 is associated with 36.6 % of the variation in the data, and PC2 with 34.1 %. Loading factors associated with PC1 and PC2 are shown in Table 4-6, however, with the inclusion of the new variable, there are no significant loading factors (> 0.6) associated with either PC1 or PC2. There is a very low correlation between dimension and PC1 (0.041), but a higher correlation (negative) between this variable and PC2 (- 0.593).

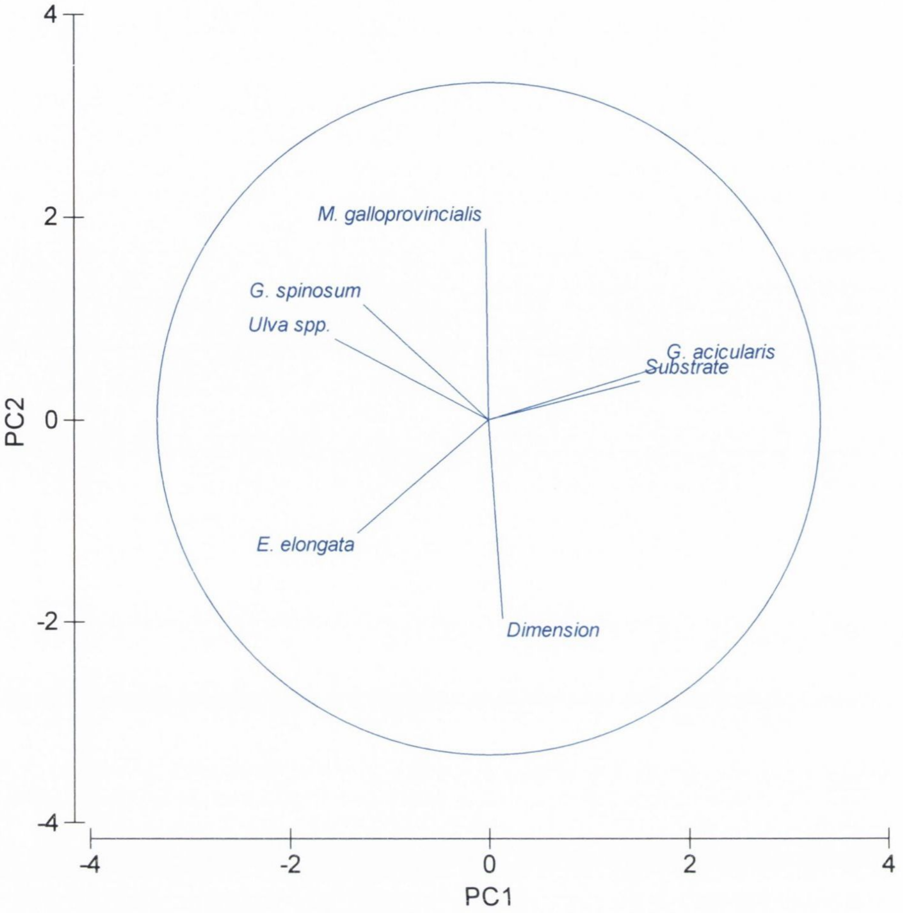


Figure 4-6 Illustrative PC biplot of data in table 4-4, with x-axis PC1 and y-axis PC2.

From the PCA biplot, dimension is largely uncorrelated with all; substrate; *G. acicularis*; *Ulva* spp. and *G. spinosum*. There is a slight positive correlation between *E. elongata* and dimension, and a negative correlation between dimension and *M. galloprovincialis.* However, regression analysis did not find a significant correlation between dimension and either *E. elongata* (r^2 = 0.1; *p* = 0.22), or *M. galloprovincialis* (r^2 = 0.3; *p* = 0.3). Thus, it is concluded that rock pool dimension did not significantly influence ecology in the current study.

4.5. Discussion

Submarine groundwater discharge rock pools were characterised by decreased salinity (as low as 4) and elevated nitrogen concentrations (up to 515μM). From extrapolation, SGD of salinity zero (i.e. the groundwater end member) was estimated to contain ~629 μM nitrogen. SGD was associated with altered structure and composition of the sessile community. In those rock pools which were used as relative 'controls', SGD was negatively correlated with *Ellisolandia elongata* cover. The rock pools surveyed varied in size and dimensions. Though previous research has suggested that larger rock pools might provide more stable conditions which may alter rock pool biodiversity (Femino and Mathieson 1980), more recent work demonstrated no significant effect of pool diameter (Underwood and Skilleter 1996) or environmental variables (tidal height, pool depth, pool width or pool length (Araújo et al. 2006) on rock pool assemblage. Principal Component Analysis identified no correlation between rock pool dimension and any ecological variable in the current study, corroborating the findings of Underwood and Skilleter (1996) and Araújo et al. (2006).

Salinity 32 psu was selected as the threshold to distinguish SGD rock pools from relative control rock pools. To examine the impact of a disturbance on a natural system it is preferable to compare disturbed sites with control sites which are (1) completely unimpacted by the disturbance, and (2) located in close proximity in order to minimise the confounding effects of external environmental factors. It is not always possible however to fully match both of these criteria, particularly in SGD ecological research. In SGD ecological research, sites in direct receipt of SGD (i.e. SGD sites) and control sites are generally located from 0.5 m to 400 m apart, depending on local conditions (Kotwicki et al. 2013; Silva et al. 2012; Migné et al. 2011; Ouisse et al. 2011; Dale and

Miller 2008). This close proximity ensures similarity of external environmental factors, but results in some influence of SGD at the relative control sites due to the inherent mobile nature of SGD. This is one of the main caveats associated with the study of the ecological effects of SGD. This frequently limits the scope of SGD ecological research, and results in the generally reduced replication in SGD research, where comparison of one SGD and one control site can occur (Migné et al. 2011). Given this caveat, it is necessary to work within the bounds of the system under consideration so that SGD ecological research might still take place. In the case where it is not possible to sample a representative control site which is completely devoid of SGD, the degree of impact of SGD can instead be established. For example, Dale and Miller (2008) sampled SGD sites and control sites along transects on an intertidal sand flat. Cores were sampled from sites located from 0.5 to 2 m apart along the SGD transect and from these, the macroinvertebrate community was investigated. They recorded a very wide range of porewater salinities associated with the sampling transects, ranging from 3.5 to 29.2 psu. Thus, all of the sites were influenced by fresh submarine groundwater discharge. To extract useful and comparative information from this data, three salinity bands were established, < 15 psu, 15 to 24 psu and > 24 psu. Ecological data were placed within one of these bands based on salinity associated with each core, and the data subsequently analysed using ANOSIM and nmMDS, similar to the study herein. Thus, where closely located but independent SGD and control sites are not available, best judgment may be used to alter the ideal experimental design so that studies of the ecology alterations associated with SGD may still be permitted. This was the case in the current study where two bands were used, 0 to 32 psu and > 32. If all rock pools with salinity lower that of the marine environment has been classified as 'sites strongly influenced by SGD', study of the ecological effects of SGD would not have been permissible in this system.

The nitrogen results in the current study are in line with those previously recorded from SGD in the area. Carvalho et al. (2013) found that intertidal SGD with salinity 4.4 contained 478 ± 14 μM N (as nitrate and nitrite) and groundwater discharging from two intertidal springs close to the platform, with salinity 2.1 and 2.4, had nitrogen concentrations of 522 ± 4 μM and 507 ± 1 3μM, respectively. Other research recorded nitrogen concentrations of up to 185 μM in SGD fed from a neighbouring aquifer but sampled on an intertidal sand flat located ~30 km east of Olhos de Água (Leote et al. 2008). The latter value is low compared to the current study however this might be explained by the non-karst aquifer type. High transport rates, low retention times and generally well-oxygenated conditions result in reduced capacity for biogeochemical reactions and nitrogen removal in karst aquifers (Slomp and Van Cappellen 2004) so that karst-channelled SGD can deliver larger fluxes of terrestrial pollutants, particularly nitrate, to the marine environment compared to other hydrogeologic settings. Coastal nitrogen loading may be associated with coastal eutrophication. The effects of the SGD nitrogen loading in the current study however were expected to be modulated by the high marine water dilution capacity in the open oceanic setting. Any ecological modifications associated with SGD nitrogen loading were expected to be restricted to the immediate receiving benthic environment, initially at least.

Measured groundwater end member nitrogen concentrations were lower than that of the most SGD-influenced rock pools and the extrapolated groundwater end member. This reflects the dynamic nature of karst aquifers where nutrient concentrations can be highly variable, both spatially and temporally. This data may suggest an accumulation of nitrate in transit through the aquifer due to nitrogen additions. This anomaly may also be due, at least in part, to the temporal separation (5 weeks) between rock pool and groundwater sampling, as the concentration of nitrogen in karst aquifers in particular varies seasonally and spatially in a non-systematic way throughout the catchment.

The decreased nitrogen concentration in the two highest salinity rock pools (~35 and ~36) relative to coastal marine water may reflect primary producer nitrogen assimilation in these rock pools in the absence of SGD-related additions. This illustrates the degree of nitrogen loading via SGD, i.e. the net nitrogen additions, or nitrogen added to the system which is in excess of that required by primary producers for fixation. Over the same time period, the biological nitrogen requirement within the highest salinity rock pools reduced the nitrogen concentration by ~12 μM, while SGD nitrogen loading to the pools increased the rock pool nitrogen concentration by ~33 to ~515 μM. Though relatively small volumes of SGD were delivered to the rock pools, the high nitrogen concentration of SGD resulted in high nitrogen loading. This has similarly been noted elsewhere with relatively small volumes of SGD causing significant ecological effects due to the high SGD nutrient (particularly of nitrogen) concentrations and loading (Valiela et al. 1990).

The structure and composition of the sessile community differed between SGD and control rock pools, and *E. elongata* was negatively correlated with the fraction of SGD in the relative control rock pools. *E. elongata* forms a fringe around mindshare rock pools, dominates the lower shore (Hayward et al. 1996, p.28) and forms turfs which can support diverse invertebrate assemblages (Bussell et al. 2007). *E. elongata* is ubiquitous in Mediterranean rock pools; a survey of 38 rock pools along 60 Km of Portuguese coast documented *E. elongata* in every pool (Araújo et al. 2006). To the best of the author's knowledge, this species has not been recorded in areas of decreased salinity. This species was found only in the relative control rock pools indicating low tolerance of SGD. Within the relative control rock pools *E. elongata* cover increased with decreasing fraction of SGD indicating high sensitivity to small changes in the proportion of SGD. In this system, SGD severely restricted and determined the distribution *E. elongata*, an otherwise normally ubiquitous species in similar fully marine systems.

Opportunistic green macroalgae (*Ulva* spp.) were the most abundant algal species, found in all but the lowest salinity SGD rock pool. Reduced-salinity-tolerant opportunistic green macroalgae have elsewhere been found associated with subtidal (Valiela et al. 1990) and intertidal (Migné et al. 2011) sites of freshwater SGD and their occurrence attributed, at least in part, to the presence of SGD (Valiela et al. 1990). *Ulva* spp. proliferate at reduced salinities, 17 – 30 psu, particularly in combination with elevated nitrogen concentrations (Kamer and Fong 2001, Martins et al. 1999). SGD in the current study was nutrient enriched and largely freshwater, as illustrated by the positive correlation between salinity and nitrogen concentration (as nitrate and nitrite). If nitrogen additions alone was the primary factor conditioning ecology in the system, the highest cover of algae, and in particular *Ulva* spp. should have been observed in the rock pool with the greatest fraction of SGD, with a positive correlation between the fraction of SGD and algal cover (and in particular *Ulva* spp.). However, the rock pool with the greatest fraction of SGD was devoid of *Ulva* spp. and there was no correlation between the fraction of SGD and the cover of *Ulva* spp.. The very depressed salinity in the rock pool with the greatest fraction of SGD may have prohibited colonisation and development of *Ulva* spp. despite the growth advantages afforded by SGD nitrogen loading. These results, combined with the negative correlation between fraction of

SGD and cover of *E. elongata* in control rock pools suggest a greater influence of salinity than nitrogen additions in conditioning the cover of macroalgal species.
Though statistically non-significant it is worth noting that, as a group, the SGD rock pools had reduced organism cover (higher bare substrate) and a reduced species list compared to the control pools. Species' introductions into rock pools depend upon recruitment from the surrounding water (Metaxas and Scheibling 1993). In the case of Olhos de Água, the surrounding water is fully marine (salinity 35), resulting in rapid dilution of SGD freshwater upon tidal inundation. SGD rock pools provide challenging conditions for marine organisms, normally intolerant of reduced salinities, however the surrounding marine environment is unlikely to harbor freshwater tolerant/estuarine organisms for recruitment to the rock pools. It is hypothesized that, despite the growth advantages associated with SGD nitrogen loading, other factors associated with SGD, including at least in part reduced salinity, caused increased bare substrate and decreased species number in SGD pools. This theory is in agreement with the theory which underpins the Venice salinity zonation system (Venice System 1959) and that of Borja et al. (2004). The ecological assemblage expected in the rock pools associated with the salinity bands/zones at the time of greatest SGD influence (~ 4 to ~ 30 psu) is distinct that ecology predicted at the salinity band/zone which occurs upon tidal influx (~ 35 psu).

The influence of SGD in conditioning the form and structure of the primary producer community has been documented elsewhere in a relatively small number of settings. SGD is often implicated in the onset and progression of eutrophication due to nutrient loading in coastal waters (Paerl 1997), particularly in areas of restricted water exchange (Dong-Woon Hwang et al. 2005; Lee et al. 2009). Johannes (1980) reviewed the tentative preliminary findings of a small number of experiments which indicated the potential significance of SGD in determining the composition and distribution of seagrass, mangrove stands and rooted salt marsh plants. Similar to the relationship between SGD and *E. elongata* documented herein, Kamermans et al. (2002) found an inverse relationship between SGD and seagrass species diversity in East African lagoons. Recruitment and proliferation of some guilds/species such as sea grasses (Kamermans et al. 2002) and *E. elongata* may be inhibited by SGD, possibly due to reduced light visibility associated with SGD-enhanced eutrophication for sea grasses, and particulars of water chemistry in the case of the latter.

In the current study, *G. spinosum* was highly correlated with ~30 % of the variation in the dataset. This variation was uncorrelated with the variation associated with *E. elongata* (which was correlated with the fraction of freshwater SGD in the control rock pools). Thus, the systematic variation associated with *G. spinosum* was due to an explanatory variable other than the fraction of freshwater SGD. A number of factors may be responsible for this variation, either entirely or in part. A number of environmental/biotic factors may alter *G. spinosum* growth rates and proliferation, including light, temperature and water motion (Macler and Zupan 1991). In the case of light, high levels of solar radiation can be a stress factor which can cause photo-inhibition of photosynthetic activity in some macroalgae (Henley 1993), including *G. spinosum* (Gómez and Figueroa 1998). Associated with this, variations in the level of shading among rock pools may explain the differences in *G. spinosum* cover between rock pools, with rock pools with greater shading supporting greater cover of *G. spinosum*. *C. mediterreanea* is less affected by the effects of high levels of photo-radiation due to its morphology. Due it its generally short length (15 – 50 mm in height (Hayward et al. 1996, p.28)), *C. mediterreanea* benefits from shading provided by taller algae and it also experiences self provision of shading due to its tuft morphology. The distribution of *E. elongata* may be less governed by shading than for other, longer non-tuft forming species, such as *G. spinosum* (usually ~80 mm in height (Hayward et al. 1996, p.26)) and *Ulva* spp. (100 to 750 mm in height (Hayward et al. 1996, p.18)). Thus light may be an explanatory variable causing systematic variation in *G. spinosum* but not *E. elongata*. The systematic variation in the distribution of *G. spinosum* may also be conditioned, at least in part, by a biotic interaction, i.e. predation and/or competition. Alternatively, the distribution/activity of some herbivore (consumer) or competitor which influences the distribution of *G. spinosum*, may be altered some biotic or abiotic factor, which alters the distribution/activity of the herbivore (consumer) or competitor, thus indirectly influencing the distribution of *G. spinosum*.

Thus, to address the original null hypotheses; (1) the sessile community differed between SGD and control (/relative control) rock pools and (2) though no correlation was observed between SGD and any ecological variable, a negative correlation was observed between fraction of freshwater SGD and *E. elongata* cover in the control(/relative control) rock pools. SGD was associated with nitrogen loading. This SGD-borne N might be of greater ecological consequence both for the immediate benthic environment and the offshore system during periods of high rainfall and/or in the future if SGD flow rate increases as domestic water needs become less reliant on groundwater sources.

To begin to derive generalisations regarding freshwater SGD in general and karst-channeled SGD in particular, the study herein is compared with a sister study conducted on the Irish west coast. The comparison considers two cases of karst-channeled SGD specifically, as flow path is the variable of perhaps greatest influence in determining the composition and flux of resulting SGD (Slomp and Van Cappellen

2004). In both systems, karst-channeled freshwater SGD is delivered intertidally, however a number of parameters differ between the two systems, limiting comparison of results and derivation of generalisations (climate, system morphology, SGD flow rate). There are, however, also some differences between the systems which enhance comparisons and provide the basis for derived generalisations (differences in intensity and form of anthropogenic catchment usage and thus SGD nitrogen load). Details of the design, results and conclusions of the Irish study are introduced here, and issues which limit generalisations derived from comparison of results are highlighted.

In contrast to the current unenclosed, exposed rock pool plateau, the Irish study was located on a rocky beach face situated at the head of a semi-enclosed bay where morphology was highly undulating and erratic with many large rocks. The semi-arid, warm temperate, Mediterranean Portuguese climate contrasts the mild, moist temperate oceanic Irish climate which results in greater aquifer recharge at the Irish relative to the Portuguese site. The Irish SGD site is covered with water of depressed salinity due to an influx of freshwater SGD water at all times, while control sites are largely exposed at low tide and covered with bay water upon tidal inundation. The Portuguese rock pools on the other hand are never exposed to the atmosphere, but, from the time of tidal retreat, a number of rock pools receive freshwater SGD, depressing rock pool salinity. It is assumed that the salinity of the rock pools returns to marine salinity upon tidal inundation due to profuse marine dilution capacity, though some rock pools may still be in receipt of SGD. Thus, the environmental conditions experienced by biota differ for both SGD sites and the relative control sites across both systems.

Quadrat surveys were conducted in both countries to characterise the composition and structure of the sessile community. As part of the Irish study, algal samples were harvested to characterise algal and invertebrate biomass and abundance. This was not carried out in Portugal due to issues around harvesting *E. elongata*. This calcareous alga forms dense tufts which adhere strongly to the substrate. A pilot study found *E. elongata* removal from rock pools arduous and highly destructive to the integrity of the plant and contained fauna. It was decided that harvesting the algae would be unrepresentative of the algal and macroinvertebrate assemblage. In Ireland SGD had salinity 0 at all times, elevated nitrogen (N) concentrations, and resulted in altered sessile community composition, similar to Portugal where freshwater SGD was N enriched and associated with altered sessile species community composition and structure. In both countries, the concentration of nitrogen in SGD was in some cases greater than that in the groundwater samples, illustrating the complex nature of karst aquifers. Though borehole groundwater values provide a good approximation of aquifer nitrogen concentration in both regions, sampling of groundwater alone may not be enough to accurately predict N loading associated with karst-channeled SGD.

The highest SGD N concentration recorded in the Irish study was ~100 µM, and in Portugal, ~515 µM. Groundwater is naturally low in nitrogen with N concentrations in excess of 160 µM indicative of anthropogenic inputs and those in excess of 600 µM of significant anthropogenic pollution (Environmental Protection Agency 2011, p.19). The European Communities Environmental Objective (Surface Waters) Regulations (S.I. No. 272 of 2209) state that at salinity 0 (i.e. freshwater), waters with < 186 µM N are of 'good status', while at salinity 34.5 (i.e. purely marine), only water with < 18 µM N merits this status, and linear interpolation using average water body salinity should be used to determine the correct values for water bodies with intermediate salinities. The nitrogen concentration of Portuguese SGD was elevated, by both groundwater and marine standards. The highest Irish SGD N concentration was below that of the groundwater thresholds, but 10 times that of the marine environment and elevated relative to the marine threshold. Comparison of these values prompts the question; what is the appropriate SGD N concentration threshold? Though there are some suggestions in the literature as to what might constitute this value, for example Mutchler et al. (2007) suggest that 30 µM N is 'N rich SGD', there is no set value. The maximum allowable concentration of phosphate in groundwater in Ireland is set by the phosphate concentration which gives rise to eutrophication in surface freshwater bodies in recognition of the connectedness between groundwater and surface freshwater bodies (Craig et al. 2010). Similarly, despite the often low salinity of SGD, because it is the marine environment where the ecological effects of the nitrogen will be felt, it seems appropriate to consider more the marine than freshwater N threshold for maximum acceptable concentrations in deriving a SGD N threshold. SGD was a source of nitrogen loading in both systems, however the concentration of nitrogen in SGD which will give rise to ecological alterations, particularly in the form of increased primary production and perhaps the initiation and onset of eutrophication in the marine environment is difficult to define due to the generally large dilution capacity in the receiving marine environment. The complications associated with this dilution capacity in terms of a SGD N threshold are illustrated by the divergent values for thresholds of N in marine and freshwater environments (S.I. No. 272 of 2209). Rather than considering SGD nitrogen concentration in terms of the standing concentrations of individual water bodies (freshwater vs. marine), it might be more appropriate to consider SGD associated N flux or loading to the marine system, which is dependent not only on the SGD nitrogen concentration but also on the volume of SGD delivered. Given the 10 x increase in nitrogen concentration in SGD compared to the background marine environment and the high SGD flux to the system (5 - 3 m^3 s^{-1} (Drew 2008)), it is suggested that the nitrogen loading associated with SGD in the Irish system is ecologically significant, despite being below the groundwater nitrogen concentration threshold.

The Portuguese rock pool with the greatest fraction of freshwater SGD contained 5 x the N concentration of Irish SGD. Differences in SGD N concentration reflect differences in catchment use, or more appropriately, intensity of catchment use. Tourism and agriculture are the main industries in both regions however both industries are relatively more intensive in Portugal than Ireland. Approximately 1 x 10^6 tourists visit the wider region of western Ireland every year, with ~18,000 people visiting the main tourist attraction in the study region, Dunguaire castle (Failte Ireland 2014). Though tourism is a main stay of the local economy in this region in Ireland, it receives far fewer tourists than the Algarve region in Portugal (the location of the Portuguese field site), which hosts ~10 x 10^6 tourists annually with average duration of stay ten days (Monteiro and Manuels 2004). The main agricultural land use in the Irish catchment is pasture and non-irrigated arable land for grazing sheep and cattle (EPA 2006). These are extensive forms of production rather than intensive (i.e. dairying, tillage, pig, poultry and horticulture) (Crowley et al. 2004). As a result, this region has the lowest agricultural productivity in Ireland and is classified as a 'marginal' farming area (Crowley et al. 2004). The Portuguese catchment on the other hand supports intensive farming, relative to the Irish catchment, and several golf courses (Cristina, et al. 2006). Agriculture on the Portuguese catchment is dominated citriculture, with horticulture of almond, fig, olive and carob trees and grape vines also important (Stigter et al. 2005). Maintaining high harvest yields of these crops requires irrigation and high fertilizer application. For example, citrus trees require 150 – 300 N kg ha^{-1} yr^{-1} (Quelhas dos Santos 1991). Intensive agriculture in coastal zones, particularly within vulnerable karstic catchment areas is linked to increased transport of contaminants (e.g., synthetic and organic fertilizer, pesticides etc.) to the coastal ocean (Coxon 2011). Groundwater bodies in many aquifers in the Algarve region of Portugal have high nitrogen concentrations, sometimes in excess of 3500 µM N (Almeida and Silva 1987), with the majority of this derived from agricultural fertilizer (Stigter et al. 1998; Stigter et al. 2009). In Ireland on the other hand, sampling of the karst aquifer groundwater ~1 km inland from the point of SGD discharge (from 2003 to 2010, sampled 2 - 4 time per year) and SGD at the study site (from 2007 to 2010, sampled 3 - 4 times per year) found groundwater mean nitrate concentration was 78 ± 30.7 µM with a range of 6 to 136 µM (Craig et al. 2010), and SGD mean nitrate 136 ± 62 µM with range 9 - 257 µM (O'Boyle et al. 2010). These results are highly congruent with the results of the current Irish study and indicate that the Portuguese catchment is subject to greater groundwater nitrogen contamination than the Irish catchment. The elevated nitrogen concentrations in SGD in both regions however illustrates the role of karst-channeled SGD in transporting nutrients even when the catchment is subject to a relatively low level of anthropogenic activities and associated groundwater nitrogen contamination. Thus, the level/relative intensity of catchment anthropogenic pressures (agriculture/tourism) and their associated stressors (particularly nitrogen loading) can be tightly coupled to SGD associated coastal nitrogen loading in the case of karst-channeled SGD.

In both countries, SGD was associated with altered species cover. In Portugal this took the form of decreased cover of *E. elongata,* and generally decreased organism cover (increased bare substrate) and decreased species diversity. In contrast, in Ireland, this took the form of increased organism coverage (reduced bare substrate) and increased species number. In the case of Olhos de Água, the surrounding water is fully marine (salinity 35 psu) with rapid dilution of freshwater SGD upon tidal inundation. SGD rock pools present challenging conditions for marine organisms, normally intolerant of reduced salinities, and the purely marine surrounding environment is unlikely to harbor freshwater tolerant/estuarine organisms for recruitment to the rock pools. In Ireland on the other hand, there was a relatively large and permanent area of reduced salinity. Surface flow (rivers, streams etc.) is absent in this karst region and all water for over 500 km^2 moves through underground caverns and conduits to Galway bay (Drew and Daly 1993) where SGD discharges at a rate of 5 – 30 m^3 s^{-1} (Drew 2008). Karst regions are generally characterised by thin soils and subsoil (Coxon 2011), which, combined with the highly permeable karst aquifer result in a very low retention time of precipitation at the surface. The fraction of precipitation which enters the coastal zone via surface runoff in this region is negligible as rainwater travels only a short distance before rapidly percolating through the thin soils, subsoils and highly permeable karst aquifer to enter the groundwater system. Thus, it is assumed that the influence of surface flow and surface runoff over the salinity of the bay is negligible and the observed reduced salinity which supports freshwater/estuarine tolerant species at the study sites is due to the SGD freshwater input. The large area of reduced salinity supported by freshwater SGD facilitates the presence of freshwater tolerant/estuarine species in the available species pool, enabling colonisation and proliferation of the estuarine algae *Fucus ceranoides*. It is suggested that the Portuguese system provided a more challenging physical environment than the Irish system due to the high degree of instability in the salinity regime.

Alterations to physicochemical parameters such as pH and concentrations of dissolved gases, particularly oxygen, can exert stress over rock pool-dewlling organisms. Though the density of the flora is the most important factor governing the range of oxygen and pH variations (Pyefinch 1943), rock pools in Portugal may experience variability in these parameters due to temperature fluctuations. Variations in temperature-dependent physicochemical parameters can induce increased stress over rock pool organisms, altering the rock pool assemblage (Pyefinch 1943). In the current system, the influence of temperature would apply equally to all rock pools alike, i.e. SGD-receiving rock pools and those not receiving SGD. The magnitude of change in temperature of the rock pools would be dependent on the volume of water in the rock pool, as smaller volumes of water will warm up quicker and thus reach greater maximal temperatures than larger rock pools which have greater water volumes. Thus, we might expect a relationship between rock pool dimension and the ecological assemblage due to variations in parameters which are a function of temperature. No correlation was observed between the ecological assemblage and rock pool dimension. However, variations in temperature-dependent parameters due to increased rock pool water temperature may be counteracted by the influx of SGD which is normally of reduced temperature relative to surface water, and particularly relative to heated rock pool water. The role of SGD mitigating variations in temperature-dependent parameters and the relevance of these mechanism in conditioning the ecological alterations associated with SGD are areas for future research.

In the current study, karst-channeled intertidal SGD was associated with altered percentage cover of the macroalgal and sessile macroinvertebrate community. In particular, SGD strongly conditioned the distribution of *E. elongata*, a normally ubiquitous species along the Portuguese coast. In Ireland, the SGD site was associated with higher coverage of the estuarine species *F. ceranoides* than the control sites, and the control sites were associated with relatively high cover of opportunistic green macroalgae compared to the SGD site. Similar to the studies considered herein, the effects of SGD on macro primary producer communities reported elsewhere have been diverse and contrasting. Kamermans et al. (2002) found no relationship between SGD and seagrass abundance or biomass in East African lagoons. Johannes (1980), however, found an inverse relationship between groundwater and seagrass species diversity, comparable to the results of the Portuguese study, but contrasting those of the Irish study. Migné et al. (2011) found an increase in primary production at an intertidal SGD site in Roscoff bay, France, congruent with the results of the Irish survey. It is suggested that individual results reflect peculiarities of individual systems associated primarily with salinity, and to a lesser extent, nutrient (N) regimes.

To date, there exist only five published studies regarding the ecological alterations associated with intertidal SGD (Dale and Miller 2008; Miller and Ullman 2004; Zipperle and Reise 2005; Migné et al. 2011; Ouisse et al. 2011). These studies document findings similar to the ones herein, with alterations in abundances of motile macroinvertebrate species previously documented in association with diffuse intertidal SGD in Cape Cod (Dale and Miller 2008; Miller and Ullman 2004), the German Wadden Sea (Zipperle and Reise 2005) and Roscoff Bay in France (Migné et al. 2011b). All five previous studies concern diffuse SGD, and none consider karst-channeled intertidal SGD. Thus, the research herein updates the current knowledge on the ecological effects of intertidal SGD in general and karst-channeled intertidal SGD, in particular. This study demonstrates that point source karst-channeled SGD alters the form and structure of the benthic intertidal food web in the receiving environment. Furthermore, by comparing karst-channeled intertidal SGD across two systems, a number of trends emerged regarding salinity, nutrient regime and broad differences in sessile community structure, adding further information regarding karst-channeled SGD to the current body of knowledge. Freshwater SGD was nitrogen enriched in both systems, demonstrating that karst-channeled SGD can deliver high nitrogen fluxes even when associated with a catchment subject to relatively low intensity of anthropogenic usage and pollution. The degree of terrestrial anthropogenic nitrogen application/pollution is closely coupled with coastal karst-channeled SGD nitrogen loading more so than for other hydrogeological settings. Though karst-channeled intertidal freshwater SGD altered the structure, diversity and composition of the sessile assemblage in both systems, the form of the alterations differed between systems. For karst-channeled SGD, depressed salinity may be of greater influence than nitrogen loading, in terms of development, form and structure of associated ecology. Given the ubiquity of karst coastlines and karst-channeled SGD, attention should be paid to the ecological effects of karst-channeled SGD, even in catchments with low intensity of anthropogenic pressure and nitrogen application.

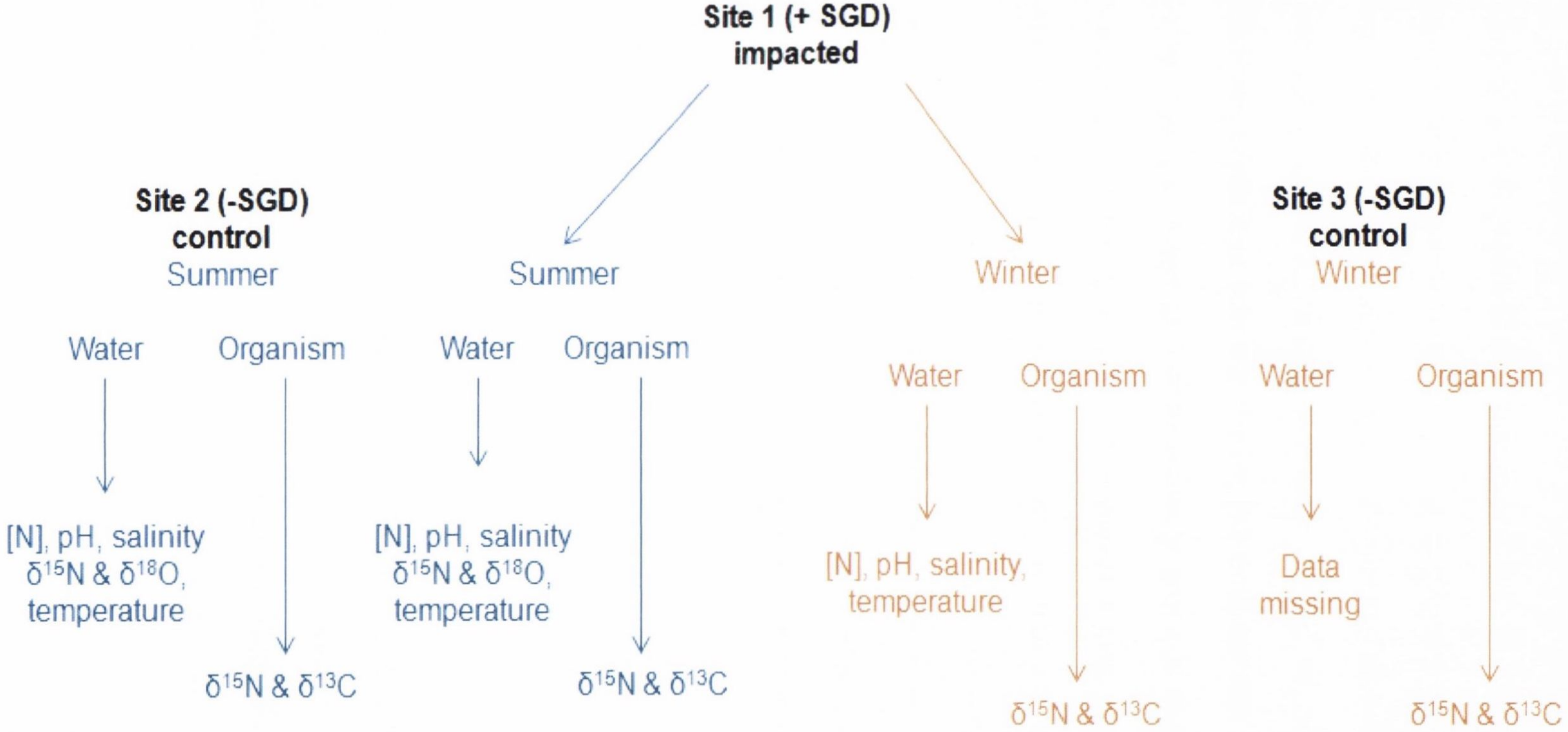
Chapter 5. Utility of stable isotope analysis (SIA) in submarine groundwater discharge (SGD) ecological research

5.1. Abstract

Freshwater submarine groundwater discharge (SGD) can cause coastal loading of nitrogen and carbon. The effects of this loading on food web dynamics and trophic transfer of SGD-borne nutrients are poorly documented, particularly for carbon. This is in part due to an absence of suitable techniques. Here, the utility of stable isotope analysis (SIA) in tracing trophic transfer of SGD-borne N and C fluxes was investigated for the first time. Furthermore, SIA was used to associate karst-channeled SGD with loading of N, and particularly C. The study was carried out in summer 2011 and winter 2012 in a semi-enclosed, sheltered bay on the west coast of Ireland, with the same one SGD site and a different control site each season. Salinity, pH and nitrogen concentrations of SGD and bay water were measured during both seasons, while nitrate isotope analysis ($\delta^{15}N_{NO3-}$ and $\delta^{18}O_{NO3-}$) was conducted in summer only. SIA of $^{12}C/^{13}C$ and $^{14}N/^{15}N$ of macroalgae and macroinvertebrates was carried out in both seasons. SGD was characterised by lower salinity and pH compared to marine samples. In winter, the concentration of nitrogen in SGD (~50 μM) was elevated compared to the control marine compartment (~16 μM), while in summer, SGD nitrogen concentration was ~8 x that of marine water (~85 μM v. ~10 μM). The $\delta^{15}N$ of SGD nitrate in summer was elevated (~2 ‰) and this was mirrored in macroalgae in summer (n.s.). In winter, the $\delta^{15}N$ of macroinvertebrates at the SGD site was also elevated ($p = 0.009$). Elevated SGD nitrogen concentration in conjunction with elevated $\delta^{15}N$ values associated with SGD (of nitrate in SGD, and organism tissue) was used to indicate SGD N loading. At the SGD site, macroalgae ($p << 0.001$) and macroinvertebrates ($p = 0.002$ in winter, n.s. for summer) were depleted in ^{13}C

In both seasons. Macroinvertebrate $\delta^{13}C$ values closely reflected that of associated macroalgae with little fractionation. The $\delta^{13}C$ of SGD macroalgae was unusually low, particularly in winter; this result, combined with depressed pH of water, was used to indicate that SGD was a source of carbon loading, supporting primary production at the SGD site. Though both $\delta^{15}N$ and $\delta^{13}C$ of biota were used as indicators of N and C loading, $\delta^{13}C$ was the more informative isotope in this system for tracing nutrient trophic transfer and discerning the influence of SGD over ecology.

5.1.1. Flow chart of Chapter 5 Experimental Design and Structure



Flow chart indicating samples collected and analysis undertaken for SGD site (site 1) and the relative control site (site 2) in summer (blue), and SGD site (site 1) and the relative control site (site 3) in winter (orange). Water samples were also taken for nitrogen concentration analysis and water chemistry analysis. These samples were collected from an inland groundwater borehole (GW) in summer and winter, and from Galway bay (GB) in winter. Stable isotope analysis of nitrate in water was also conducted on the summer groundwater sample (GW).

5.2. Introduction

Karstic and carbonate geologies comprise some 25 % of the world coastlines (Ford and Williams 2007) and freshwater SGD frequently occurs where karst aquifers are hydraulically connected to the sea (Fleury et al. 2007). Freshwater SGD can provide a pathway for anthropogenic nutrients to the marine environment (Slomp and Van Cappellen 2004), and is frequently associated with coastal nutrient loading (Hwang et al. 2010; Waska and Kim 2010b; Corbett et al. 2000; Burnett et al. 2007; Niencheski et al. 2007; Leote et al. 2008). Freshwater SGD, particularly, is associated with loading of nitrogen, the nutrient which often limits marine primary productivity and is thus of concern in terms of eutrophication (Howarth and Marino 2006; Nixon 1995). SGD may also deliver dissolved inorganic carbon (DIC) to the coastal water column in carbonate coastal areas. Dorsett et al. (2011) estimated that 7 – 11 % of global coastal water DIC may be derived from SGD associated with karst and other carbonate systems. There is a lack of data and research on the interrelation between coastal food webs and SGD-borne nutrients. This is due to a number of factors, namely (1) the temporal and spatial variability inherent in SGD; (2) difficulties around identifying SGD when it is subtle, i.e. flow rate is low or outflow diffuse in nature; and (3) an absence of suitable techniques for detecting food web utilisation of SGD-borne nutrients.

The occurrence of SGD can be spatially and temporally variable, rendering its location, identification and characterisation difficult. For example, in Ireland alone, over 35 sites of freshwater SGD are postulated (Wilson and Rocha 2012), however only two of these have been verified (Wilson and Rocha 2012; Drew and Daly 1993). Due to temporal and spatial variability, SGD induced ecological alterations may not be as pronounced as for other more sustained sources of nutrient loading. The ecological alterations may however be progressive over time due to cumulative effects of nutrient loading. Such gradual ecological alterations may not be readily perceptible to the naked eye or detectible during periodic routine sampling, negating their scientific investigation. Finally, there is an absence of suitable techniques in this area of SGD research. Techniques are needed to identify this allusive process and associate it with food web utilisation of SGD-borne nutrients, in spite of the aforementioned issues. One technique which may potentially prove useful in the study of ecological aspects of SGD is stable isotope analysis (SIA). The analysis of nitrogen ($^{15}N/^{14}N$), oxygen ($^{18}O/^{16}O$) and carbon ($^{13}C/^{12}C$) stable isotope ratios are commonly used in many fields of research, including marine ecology and pollution.

The $\delta^{15}N$ value of nitrate in water can be indicative of the source of the nitrate, and can in particular differentiate natural from anthropogenic origins. The $\delta^{15}N$ value of synthetic fertilizer generally ranges from - 2 to + 4 ‰, of soil organic nitrate from + 2 to + 6 ‰ with an average of + 4 ‰ (Xue et al. 2009), of relatively untreated human/animal waste from + 5 to + 9 ‰ (Mutchler et al. 2007), and of treated animal/human waste from + 10 to + 38 ‰ due to the effects of denitrification (Gartner et al. 2002). The $\delta^{15}N$ value of nitrate in groundwater which is not anthropogenically nitrogen contaminated tends to range from + 2 to + 6 ‰ with an average of ~+ 5 ‰, and that of uncontaminated surface water from + 1 to + 6 ‰ with an average ~+ 4 ‰, in both karst coastal (Mutchler et al. 2007) and non-karst (Xue et al. 2009) systems.

The $\delta^{18}O$ value of microbially-produced groundwater NO_3^- is determined by the $\delta^{18}O$ values of H_2O and atmospheric O_2 as, in theory, two oxygen atoms of the newly formed NO_3^- derive from H_2O and the third atmospheric O_2 (Xue et al. 2009). The $\delta^{18}O$ value of atmospheric oxygen is + 23.5 ‰, that of marine water ~0 ‰, and that of fresh groundwater is lower (~- 6 ‰) due to isotopic fractionation associated with evaporation and subsequent phase changes prior to precipitation. The $\delta^{18}O$ value of marine nitrate ranges from + 2 to + 20 ‰ and that of nitrate fertilizer ranges from + 18 to + 25 ‰, while that of nitrate contained in soil and animal/human waste produced via nitrification ranges from - 16 to + 15 ‰ (Kendall et al. 2007, p.381; Xue et al. 2009).

Many biologically-mediated processes consist of a number of steps (e.g. nitrification: $NH_4^+ \rightarrow NO_2^- \rightarrow NO_3^-$). Each step has the potential to cause isotopic fractionation but the largest degree of fractionation is generally associated with the rate-determining or slowest step. This step usually involves a relatively large pool of substrate; the amount of which that reacts is small compared to the initial substrate reservoir. In contrast, a step that is not rate-determining generally involves a small pool of a substrate, all of which is rapidly converted to product therefore supporting little/no net fractionation (Kendall et al. 2007, p.394). Nitrification does present a fractionation interval for oxygen as the reaction does not go to completion, i.e. not all substrate (ambient oxygen) is converted into product (nitrite and then nitrate) and it involves a relatively large pool of substrate. That is, the amount of which that reacts is small compared to the initial substrate reservoir. Though this is a fractionating step for nitrogen, in well oxygenated systems it does not result in any net fractionation as the reaction goes to completion, i.e. all of the initial substrate pool (ammonia) is converted to product (nitrite and then nitrate), thus not imparting nitrogen isotopic fractionation. Thus, the O and N isotopic signature of nitrate in water can indicate the original nitrogen source, and inform on the O source.

The dissolved inorganic carbon (DIC) in freshwater is depleted in ^{13}C relative to marine DIC providing a tool for distinguishing freshwater- and marine-based food webs. The

$\delta^{13}C$ value of total DIC in the ocean ranges from 0 to + 2 ‰ (Zeebe and Wolf-Gladrow 2001, p.168), while groundwater DIC in carbonate regions ranges from - 17 to 0 ‰ (Zeng and Masiello 2008; Dorsett et al. 2011; Spiker 1980). The main sources of carbon to freshwater; atmospheric CO_2, carbonic acid dissolution of carbonates and respired CO_2 (Kendall et al. 2001) provide DIC which is depleted in ^{13}C. The $\delta^{13}C$ value of atmospheric CO_2 ranges from -14.4 to -8.4 ‰ (Górka et al. 2011), but when freshwater is in equilibrium with atmospheric CO_2 at pH >7, the $\delta^{13}C$ value of DIC is near 0 ‰. The $\delta^{13}C$ value of DIC produced from the dissolution of carbonates generally ranges from -15 ‰ to 0 ‰ (Kendall et al. 2001; Brunet et al. 2005). Carbon respired by algae usually reflects the signature of the algae (Kendall et al. 2001), which normally ranges from -3 to -35 ‰ (Raven et al. 2002). Thus the $\delta^{13}C$ values of DIC in groundwater generally range from -5 to -25 ‰ (Kendall et al. 2001), and of groundwater in carbonate regions from 0 to -17 ‰, relative to marine ~0 ‰.

The isotopic signatures of C and N change in relatively predictable ways as they enter and move through the food web. Herbivores become enriched in ^{13}C by ~0 ‰ to 1 ‰ relative to their primary producer source (DeNiro and Epstein 1978; Peterson and Fry 1987), and similarly generally enriched in ^{15}N by ~2.5 ‰ to 3.4 ‰ (Zanden and Rasmussen 2001; Vanderklift and Ponsard 2003; DeNiro and Epstein 1981). The $\delta^{13}C$ and $\delta^{15}N$ of primary producers reflect that of the available nutrient pool with variable fractionation, proportional to the excess of nutrient supply relative to demand (Marshall et al. 2008, p.22; Mariotti et al. 1981). These attributes permit investigation of food web dynamics, particularly in terms of determining N and C sources (Currin et al. 1995; McClelland et al. 1997) and tracing the trophic transfer of these nutrients.

A number of factors will modulate $\delta^{13}C$ value of aquatic plants including depth (Fischer and Wiencke 1992), turbulence, growth rate and the factor limiting growth (Cassar et al. 2006). The most consistent and influential factor however is the concentration of dissolved CO_2, and associated with this, the presence or absence of various forms of carbon concentrating mechanisms (CCMs) and resulting differential use of CO_2 and HCO_3^- (Fry and Sherr 1989; Kerby and Raven 1985; Maberly et al. 1992; Lehmann et al. 2004). Algal carbon isotopic fractionation is predominantly controlled by carbon fixation during photosynthesis (Kerby and Raven 1985; Lehmann et al. 2004). CO_2 diffuses across the cell membrane passively, while HCO_3^- must be first converted to CO_2 extracellularly via carbonic anhydrase before diffusion (Axelsson et al. 2000). Therefore, though HCO_3^- is the dominant species of inorganic C in seawater, CO_2 use is more energy efficient. There is considerable fractionation of carbon isotopes between the species of the carbonate system, i.e. isotopes are not distributed equally among CO_2, HCO_3^-, and CO_2^-. For example, at pH 7 - 8 and salinity 35 psu, the $\delta^{13}C$ value of CO_2 is ~-9 ‰ while that of HCO_3^- is ~0 ‰ (Zeebe and Wolf-Gladrow 2001, p.182). This is due largely to fractionation during dehydration of HCO_3^-, i.e. $H^+ + HCO_3^- \rightarrow CO_2 + H_2O$ (Zeebe and Wolf-Gladrow 2001, p.182). Resulting from this, the $\delta^{13}C$ value of the CO_2 fraction of DIC can be more negative than the HCO_3^- fraction by 10 ‰ (Maberly et al. 1992) to 22 ‰ (Zeebe and Wolf-Gladrow 2001, p.182), though the usual range is 8 to 11 ‰ (Lehmann et al. 2004). Due to enrichment of ^{13}C in HCO_3^- relative to CO_2, there are clear differences in the $\delta^{13}C$ values of algae that can use HCO_3^- (- 8.81 to - 22.55 ‰) and those that only use CO_2 (- 29.90 to - 34.51 ‰) (Maberly et al. 1992).

The influence of season over primary producer CO_2 demand and dissolved $[CO_2]$ temporally conditions algal $\delta^{13}C$ values. Algal $\delta^{13}C$ values are largely a function of primary producer CO_2 demands for fixation relative dissolved CO_2 supply/availability (i.e. the concentration of dissolved CO_2 (hereafter [CO_2 (aq)])). High primary producer C demand relative to availability results in increased reliance on HCO_3^- (which is ^{13}C enriched relative to CO_2) and reduced apparent fractionation between the organic matter (primary producer) pool and CO_2, causing primary producer enrichment in ^{13}C and elevated $\delta^{13}C$ values (Lehmann et al. 2004; Mook et al. 1974). When primary producer CO_2 demand is low relative to availability, there is less reliance on HCO_3^- and greater apparent fractionation between the organic matter pool and CO_2, causing primary producer depletion in ^{13}C and lower $\delta^{13}C$ values. As cold water can hold more CO_2 than warm water (Matsuoka et al. 2001), water [CO_2 (aq)] is higher in winter than summer. Also, reduced photosynthesis associated with lower temperatures and reduced photon flux in winter leads to reduced winter primary producer CO_2 requirements (Wiencke and Fischer 1990). These changes in primary producer C assimilation rate and fluctuations in the availability of dissolved CO_2 associated with seasonality cause temporal changes in primary producer $\delta^{13}C$ values (Lehmann et al. 2004). High water [CO_2 (aq)] relative to primary producer DIC demand results in winter algal ^{13}C depletions (Lehmann et al. 2004), with values as low as - 30 ‰ recorded (Wiencke and Fischer 1990).

During photosynthetic fixation of CO_2 into organic matter, primary producers discriminate against the heavier isotope so that algal material is isotopically lighter than its CO_2 source and the DIC pool becomes progressively ^{13}C enriched (Burkhardt et al. 1999). Based on this, the $\delta^{13}C$ values of autochthonous organic matter can be used as an indicator of surface water [CO_2 (aq)], normally a logarithmic relationship, in freshwater systems (Lehmann et al. 2004; Gu et al. 2011). For example, Lehmann et al. (2004) measured the $\delta^{13}C$ of particulate organic carbon (POC) and [CO_2] in a mesotrophic lake in Switzerland and found a good logarithmic correlation between the two variables (Figure 5-2). This relationship may be adapted to the current study and algal $\delta^{13}C$ values used to indicate the role of SGD in C loading.

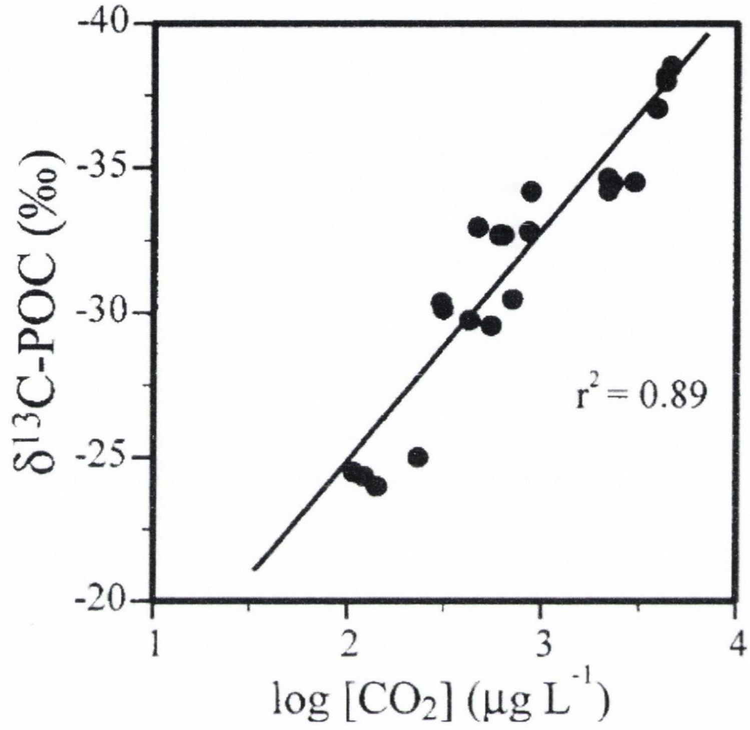


Figure 5-1 Correlation between surface water (0 - 5 m) [CO_2 (aq)] and the $\delta^{13}C$ values of POM in a high productivity lake (Lake Lugano, Switzerland). Adapted from Lehmann et al. (2004).

Four previous studies of SGD employed SIA of organism tissue (Kamermans et al. 2002; Mutchler et al. 2007; Ouisse et al. 2011; Carruthers et al. 2005). Three considered algal $\delta^{15}N$ values (Kamermans et al. 2002; Mutchler et al. 2007; Carruthers et al. 2005) and one considered algal and macroinvertebrate $\delta^{13}C$ and $\delta^{15}N$ values (Ouisse et al. 2011). Three looked at the effect of SGD-borne wastewater on select species of primary producers (Kamermans et al. 2002; Carruthers et al. 2005; Mutchler et al. 2007) and the fourth considered the effect of SGD on food web structure (Ouisse et al. 2011). While these studies provide interesting results, none investigated the application of SIA in (1) identifying food webs which utilise nutrients derived from SGD and delineating SGD and control food webs or (2) describing the utilisation of SGD-borne N and C by coastal food webs in the case of relatively anthropogenically uncontaminated (i.e. low N load relative to groundwater pollution thresholds) freshwater intertidal SGD.

The current study was based on the premise that the δ^{15}N and δ^{13}C of SGD and marine water differ. Thus the δ^{15}N and δ^{13}C values of SGD and control food web components should differ due to utilisation of different C and N sources. It was predicted that, due to the effects on SGD water chemistry of the karst system, δ^{13}C values would provide the more robust tracer for delineating food webs. Different sample compartments (water, primary producers, consumers) reflect different (increasing, respectively) time frames of integration of source isotopic signature. Thus it was expected that sampling all three compartments would provide a more comprehensive picture and allow discrepancies due to short term shifts in signature to be de-convoluted.

The aims of this study were to assess the utility of stable isotope analysis in the study of SGD, and specifically, assess the application of SIA in (1) tracing the trophic transfer of SGD-borne N and C, enabling discernment of food webs in direct receipt of SGD from relative control food webs, and (2) determining if karst-channeled intertidal freshwater SGD is a source of N and C loading in this system. To address these aims, it was hypothesized that (1) the δ^{15}N values of macroalgal and macroinvertebrate food web components from SGD and control sites would differ and reflect that of the basal SGD/marine water nitrate source respectively, and (2) the δ^{13}C values of SGD algae and macroinvertebrates would differ from that of the control food web. To investigate these hypotheses, the following null hypotheses (Ho) were tested:

Ho(1): The nitrogen isotopic signature of SGD food web components (macroalgae and macroinvertebrates) will not differ from that of their food web counterparts.

Ho(2): The carbon isotopic signature of SGD food web components (macroalgae and macroinvertebrates) will not differ from that of their food web counterparts.

Water chemistry parameters ($\delta^{15}N_{NO3-}$, $\delta^{18}O_{NO3-}$, pH, nitrogen concentration, salinity and temperature) and organism C and N elemental data were also assessed to complement the organism isotopic data.

5.3. Methods

5.3.1. Study area

The study site, Kinvara bay, is situated on the mid-western coast of Ireland (53° 8' 22.79" to 53° 10' 35.08" N and 008° 56' 10.34" to 008° 57' 50.45" W). The bay is ~4 km in length and 2 km at its widest point, though more than half of the bay is less than 1 km wide. It is a smaller inlet of the larger Galway bay (53° 14' 5.32" to 53° 7' 54.99" N and 008° 58' 64" to 009° 34' 22" W) (Figure 5-3). There is a well documented locus of intertidal discharge at the head of Kinvara bay (Drew and Daly 1993; Drew 2008). The karst aquifer which feeds SGD into the bay is underlain by a Carboniferous (Viséan, ICS (International Convention on Stratigarphy) timescale) limestone bedrock (O'Connor et al. 1993). Mean annual precipitation ranges from 1000 – 1400 mm, with monthly precipitation ~150 mm in winter and ~50 mm in summer (Kiely 1999). Approximately half of this precipitation becomes groundwater recharge (Drew, 2008). With an absence of surface flow, all water for over 500 km^2 moves through underground caverns and conduits to Galway bay (Drew and Daly 1993) where SGD discharges at a rate of 5 – 30 $m^3 s^{-1}$ (Drew 2008). Salinity within Kinvara bay ranges from 0 - 33 psu and temperature from 11.2 °C to 16.7 °C (Kiely 1999). Generally warm summers and mild winters characterise the cool temperate maritime climate (Kiely 1999).

Kinvara village, the main urban agglomeration in the area is located from ~600 - 800 m from the locus of SGD. In 2011, Kinvara village and the surrounding town land of Gort had a combined population of 2,644 people (CSO 2011) with 70 % of those living in rural areas (McGovern 2012). The bay is a natural oyster bed (Cannon 2010), the site of a mussel aquaculture industry and the focus of recreational activities vital to tourism in the region. Tourism, which provides the main source of employment (Gallagher et al.

2010) brings a concomitant influx of pollution to the area which is transferred into the bay as sewage. Some 315 m^3 of untreated sewage enter Kinvara bay through a point source outflow pipe every day (Cannon 2010, p.166) (Figure 5-3). Agriculture is the main primary industry in the region (Gardiner and Radford 1980) with agricultural land used mostly for pasture and non-irrigated arable land for sheep and cattle grazing (EPA 2006). Due to the grassland-dominant agriculture and a low level of industry and urbanisation, groundwater pollution derives mostly from farming activities and septic tank effluent (Thorn and Coxon 1992). Pollutants include nutrients of eutrophication concern (phosphorus and nitrate), pesticides, antibiotics, and microbial pathogens (Coxon 2011).

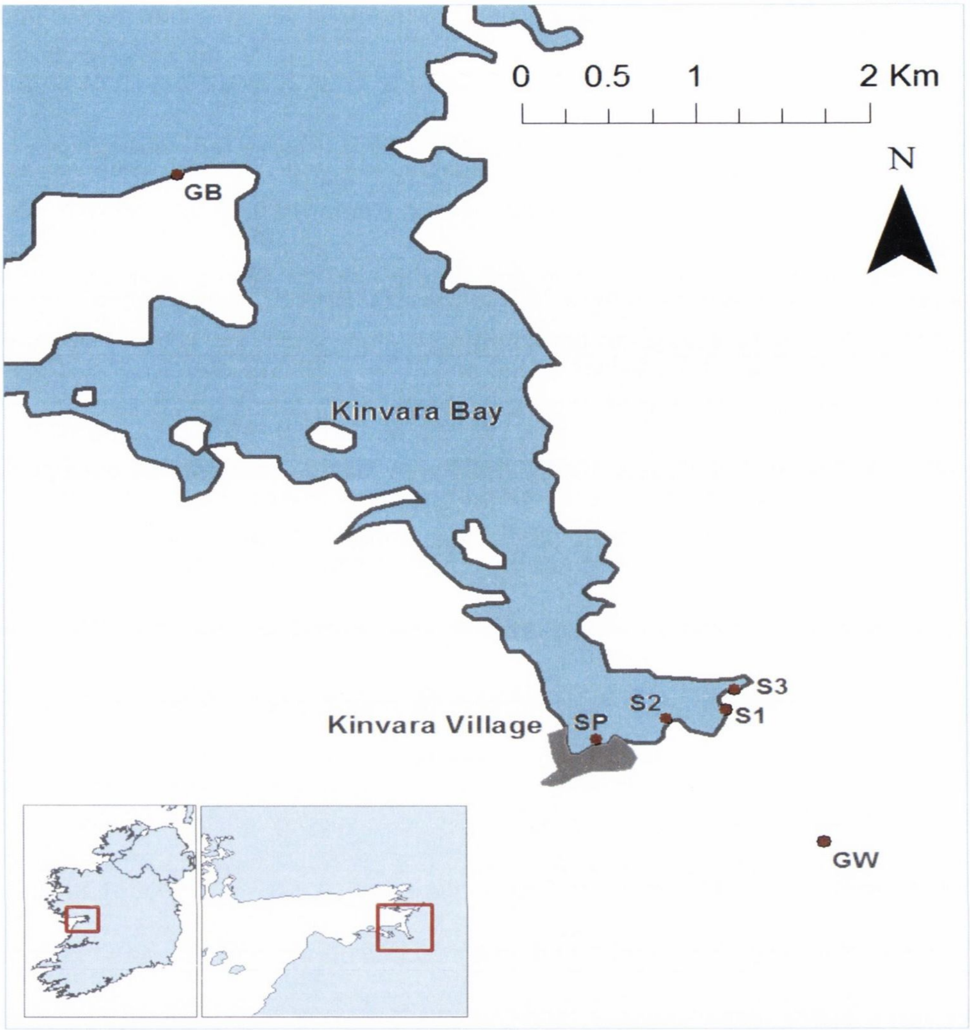


Figure 5-2 Study site and sample locations. Galway Bay and smaller inlet, Kinvara bay, indicating sampling locations; Galway bay (GB), SGD site (S1), and summer and winter control sites (S2 and S3, respectively). Also indicated are the locations of the inland groundwater sample borehole (GW), and sewage outflow pipe (SP).

5.3.2. Study site selection

Site 1 (common to both seasons) was chosen based on the previously documented presence of focused SGD (Drew 2008), and located at point the of SGD entry into Kinvara bay. The summer control site (site 2) was located to the west of site 1, and the winter control site (site 3) to the east on the far side of a rocky outcrop (Figure 5-3). Sites 2 and 3 distanced ~300 meters and ~100 meters from site 1, respectively. As is normal in SGD ecological research, control sites were located in close proximity to the SGD site (Kotwicki et al. 2013; Silva et al. 2012; Migné et al. 2011b; Ouisse et al. 2011) to reduce variation in external factors. This however lead to some influence of SGD at the control sites so that control sites were 'relative control' sites rather than absolute control sites devoid of the influence of SGD. Site 1 was covered with water (SGD) at low tide as well as high tide. At low tide, site 3 was completely exposed to the atmosphere while trickles of water of reduced salinity (12 psu) were a feature of some sections of site 2. A sewage outflow pipe is located ~750 meters to the west of site 1 (Figure 5-3). It was desirable for site 2 to be distant from this pipe but located on the same side of the bay as site 1 to increase comparability of sites.

5.3.3. Water sampling and in-situ parameters

As control site 3 was completely exposed to the atmosphere while trickles of water of reduced salinity (12 psu) were a feature of some sections of site 2, water samples were collected at control site 2 at low tide, but not at control site 3. Water samples were collected from; site 1 and a groundwater borehole at 70 m depth located ~1 km inland of site 1 in both years; site 2 (control) in summer; the marine environment directly offshore of site 1 and site 2 at ~15 cm depth in summer ('SGD/marine' and 'control/marine' respectively); and Galway bay at a site located close to the mouth of Kinvara bay in winter. Samples were collected in 500 ml or 1000 ml acid-washed polyethylene (PE) bottles. One PE bottle was taken at each site, and from this 2 - 4 individual replicate water samples were filtered for analysis of nitrogen concentration. Where relevant, 2 replicates were filtered for nitrate isotopic determination. For all samples, except for site 2 where water depth was insufficient, bottles were immersed closed and opened under water to collect the samples. At site 2, the open bottle was held just above the sediment with the open end perpendicular to the direction of water flow to collect the sample. Approximately 75% of the volume of the bottle was immersed in this position, enabling water sampling.

Samples for determination of nitrogen concentration were immediately filtered through poly-ether-sulphone (PES) membranes (Rhizon SMS-10 cm; Eijkelkamp Agrisearch Equipment 0.1 µm pore size) into sterile 11 ml vacuum tubes (BH vacutainer) via a needle connected to the membranes via tubing. The first 5 ml of each sample was discarded as dead volume. Collection via this method precludes the need for further preservation (Luo et al. 2003; Seeberg-Elverfeldt et al. 2005). Samples were stored at 4 °C until analysis. Water samples were analysed for combined NO_3^- and NO_2^- via flow injection analysis (FIA) on a Lachat™ QuickChem 8500 instrument following the dual determination of nitrite and nitrate analysis using a cadmium column method (Anderson 1979; Johnson and Petty 1983).

Samples for determination of water nitrate isotopic signature ($\delta^{15}N$ and $\delta^{18}O$) were taken in summer only. Samples were filtered through 0.7 µm pre-combusted (450 °C for 4 hours) GF/F filters into 100 ml sterile PE bottles. Samples were stored on ice in the dark and transferred to -70 °C within 4 hours of collection. Salinity, pH and temperature were measured *in-situ* using a WTW Cond197i multi parameter probe and GPS location recorded with a Garmin™ eTrex handheld GPS navigator.

5.3.4. Macroalgae and macroinvertebrate field sampling

To address null hypotheses (1) and (2), nitrogen and carbon stable isotope analyses were carried out on macroalgal and macroinvertebrate samples from the SGD and control site. Sampling was conducted during low spring tide. At each site (1, 2 and 3) a 5 m^2 study station was delineated. As there was no visible vertical shoreline zonation, stations were located at approximately the same shore height as determined by measuring the distance from the seaward end of the sampling station to the water at low tide (~25 m). Random number tables were used to determine the location of five sample sites, marked with 0.5 m^2 quadrats, within each station. *Fucus ceranoides* (Linnaeus, 1753) (top 2 cm tip of plant) and *Ulva* spp. (Linnaeus, 1753) (all biomass present in quadrat) macroalgae were harvested from within quadrats for subsequent $\delta^{15}N$ and $\delta^{13}C$ isotopic analysis. In summer, three replicate samples of *Fucus ceranoides* were sampled from each of four quadrats, and two replicates of *Fucus ceranoides* from the fifth quadrat at both sites 1 and 2 (i.e. n = 14). All biomass of *Ulva* spp. contained within a quadrat was harvested and pooled to give one *Ulva* spp. sample per quadrat; this amounted to two samples for site 1 and five samples for site 2. Therefore, n = 16 for site 1 and n = 19 for algal isotopic analysis for site 2 in summer. In winter, three replicate samples of *F. ceranoides* were sampled from each quadrat in addition to two samples of *Ulva* spp. from site 1 and one sample from site 3. The analysis effort was reduced for algae in winter to accommodate reduced resources. Thus, for *F. ceranoides* in four quadrats, the three replicate samples were pooled for isotopic analysis, and for the fifth quadrat, all three samples were analysed. The composite *F. ceranoides* sample from quadrat two in site 3 was lost during processing. Therefore, for winter algal isotopic analysis, n = 9 for site 1 and n = 7 for site 3 (see Appendix E for an explanatory table). All algae were placed in plastic Ziploc™ bags and returned to the laboratory on ice. From within each 0.5 m^2 quadrat, a 15 cm^2 area of all algal biomass was harvested into a Ziploc™ plastic bag with a scraper and transported to the lab on ice. Macroinvertebrate samples for $\delta^{15}N$ and $\delta^{13}C$ isotopic analysis were removed from the macroalgal biomass to address null hypothesis (1) and (2). Blue nitrile gloves were worn throughout sampling to prevent sample contamination.

5.3.5. Macroalgal and macroinvertebrate laboratory processing

Algal samples were rinsed in de-ionised water and scraped to remove epiphytes. Each sample was then acidified with 1M HCl to remove any remaining sand/carbonate particles, washed with de-ionised water, placed in Ziploc^{TM} plastic bags, and retained at - 70 °C for transport back to the main laboratory for further processing. Harvested algal biomass samples were rinsed with filtered seawater in a 1 mm sieve to remove larger particles and organisms, and the remaining sample sorted through. All visible macroinvertebrates were removed, identified to the lowest possible taxonomic level using a dissecting scope, and counted. Pooled samples of the same species from a given quadrat were held overnight in filtered seawater to depurate gut contents. Individuals of the same species for the same sample placed in labeled Ziploc™ bags and held at - 70 °C until further processing. As is common ecological studies involving small organisms, the entire organisms were used for stable isotope analysis (e.g. Hsieh et al., 2002; Riera et al., 2004; Kang et al., 2006).

Algal and macroinvertebrate samples were places in an oven at 60 °C for 48 hours to ensure complete desiccation. Samples were then manually ground to a fine homogenous powder using an agate mortar and pestle. All equipment was thrice cleaned with ethanol and allowed to air dry between samples. Ground, desiccated macroinvertebrate samples were acidified with 1 M HCl, drop by drop until effervescence stopped to remove carbonates which might otherwise alter the $\delta^{13}C$ isotopic signature (as per Vizzini and Mazzola, 2003; Carabel et al., 2006; Colombini et al., 2011). After effervescence ceased, samples were immediately transferred to a 60 °C oven for 48 hours to re-dry. All ground samples were stored in a desiccator until stable isotope analysis.

5.3.6. Stable isotope and elemental analysis

Bulk sample δ^{15}N and δ^{13}C analysis of macroalgal and macroinvertebrate tissue was conducted to test null hypotheses (1) and (2) respectively. Furthermore, samples collected in winter were subject to elemental analyses to determine %C, %N and C:N. This was not conducted for winter samples as it was not standard practice to collect this data at the time of analysis and it was not possible to obtain the data retrospectively. For this, macroalgal material (2.0 - 3.5 mg) and macroinvertebrate material (2.0 - 4.1 mg) were weighed into tin capsules using a microbalance. Analysis of δ^{15}N and δ^{13}C was carried out using a Thermo-Finnigan Delta V Advantage IRMS online with a Costech Elemental Analyser (EA) at the University of Durham (SIBL - Stable Isotopes in Biogeochemistry Laboratory) for samples collected in summer. Samples collected in winter were analysed for δ^{15}N and δ^{13}C on an ISOPRIME 100 IRMS (Isoprime Corporation, Cheadle, UK) and a vario ISOTOPE cube elemental analyzer (Elementar Analysensysteme GmbH, Hanau, Germany) at the Helmholtz Centre for Environmental Research – UFZ, Leipzig, Germany.

Isotopic signatures are reported using δ notation in permil (‰) deviations from the standards, atmospheric nitrogen (N_2), Vienna PeeDee Belminite Limestone (V-PDB) for carbon, and Vienna Standard Mean Oceanic Water (V-SMOW) for oxygen. Isotopic signatures were calculated and are expressed as;

$$(R_{sample} - R_{\mathrm{standard}})/R_{standard}) \times (1000)\ \delta\ ‰$$

where R is the ratio of heavy to light isotope, R_{sample} is the sample ratio, and $R_{standard}$ that ratio in the standard (Sulzman 2007, p.6).

Blue nitrile gloves were worn at all stages of laboratory processing and all equipment cleaned with ethanol and allowed to air dry between samples. Analytical error, derived from the standard deviation of replicate measurements of standards, was 0.15 ‰ for $\delta^{15}N$ and 0.46 ‰ for $\delta^{18}O$ for nitrate in water analysis, 0.1 ‰ for both $\delta^{13}C$ and $\delta^{15}N$ for the analysis of organisms collected in summer, and 0.4 ‰ for $\delta^{15}N$ and 0.2‰ for $\delta^{13}C$ for the analysis of those organisms collected in winter.

Samples for determination of $\delta^{15}N$ and $\delta^{18}O$ of NO_3^- in water were posted frozen to University College Davis (UCDavis), University of California, U.S.A. There, using the bacterial denitrifier method (Sigman et al. 2001), NO_3^- was bacterially denitrified to N_2O, the $\delta^{15}N$ and $\delta^{18}O$ of which was subsequently analysed. Following denitrification, N_2O gas samples were purged from vials through a double-needle sampler into a helium carrier stream (25 mL min^{-1}). Samples were then passed through a CO_2 scrubber (ascarite) and N_2O trapped and concentrated in 2 liquid nitrogen cryo-traps. The samples then passed to a second trap which was warmed to ambient temperature, and the N_2O carried by helium to the IRMS via an Agilent GS-Q capillary column (30 m x 0.32 mm, 40°C, 1.0 mL min^{-1}). $\delta^{15}N$ and $\delta^{18}O$ values were measured using a Thermo-Finnigan Gas-Bench and Pre-Con trace gas concentration system interfaced to a Thermo-Scientific Delta V Plus Isotope Ratio Mass Spectrometer (IRMS) (Bremen, Germany).

5.3.7. Data analysis

To accommodate small samples sizes and lack of normality (Anderson-Darling test: $p < 0.05$) in some cases, Welch's T-tests or non-parametric Wilcoxon rank sum tests were used, as appropriate, for comparisons. Welch's T-test is a recommended parametric alternative to Students T test where unequal sample sizes are being compared (Myers et al. 2013, p.138) and similarly the Wilcoxon rank sum test can handle unequal sized samples (Fraunhofer 2009, p.179). All statistics were conducted using R (R Development Core Team 2011), and a significance level 95% ($\alpha = 0.05$) was set for all tests. The *nortest* package in R was used for Anderson-Darling tests and all other tests were inherent in R and not required to be loaded as packages.

5.4. Results

5.4.1. Water chemical parameters and nitrogen concentration

Table 5-1 Water physicochemical data; salinity (psu), pH and NO_x (NO_3^- + NO_2^-) concentration, and isotopic composition of nitrate in water data for SGD sampled from site 1, the control site 2 (site 2), groundwater and Galway bay in each season, where relevant.

						NO_3^-	
Season	**Sample**	**Salinity(psu)**	**pH (1SE)**	**Temperature (°C)**	**NO_x (1SE)(μM)**	**$\delta^{15}N$ (SE, n = 2)**	**$\delta^{18}O$ (SE, n = 2)**
Summer	GW 2011	0	7.21	16.4	66.7 (1.1)	7.95	16.38
	SGD 2011	0	7.06 (0.15)	11.9	59.7 (1.1)	8.57 (0.05)	16.64 (0.18)
	SGD/marine	0	7.07	11.2	61.4 (1.3)	8.15 (0.00)	17.99 (0.15)
	Control site 2	12.1	8.05 (0.11)	15	15.6 (0.1)	6.63 (0.7)	18.48 (0.15)
	Control/marine	-	-	-	29.1 (0.8)	6.32 (0.03)	17.47 (0.14)
Winter	GW 2012	0	7.4	11.6	60.2 (0.1)		
	SGD 2012	0	7.3 (0.16)	11.3	90.6 (3.6)		
	Galway bay	33	8.2	10	10.2 (0.1)		

All SGD samples (including the SGD/control sample) and groundwater samples had salinity 0 psu. The SGD and the SGD/marine samples had salinity 0 and similar NO_x concentration, indicating a lack of mixing of freshwater SGD with marine water at the point of SGD/marine water sampling. The salinity of water from site 2 was 12 psu, and that of Galway bay 33 psu. The pH of SGD samples and groundwater was lower than that of site 2 water and the Galway bay water by ~1 unit (Table 5-1). The water occurring on some sections of the summer control site had a salinity of 15 psu, and that of the Galway bay sample was 33 psu. In both seasons, the pH of groundwater and SGD was comparable, and lower than that of either the control site 2 or Galway bay water by ~1 unit. SGD temperature was approximately the same (~11 °C) in both seasons. The temperature of SGD and control (S2) water were comparable in summer. Galway Bay water was colder than SGD by ~1 °C in winter (Table 5-1). In winter, the temperature of groundwater was comparable to that of SGD. In summer however, groundwater was ~4 °C warmer than that of SGD

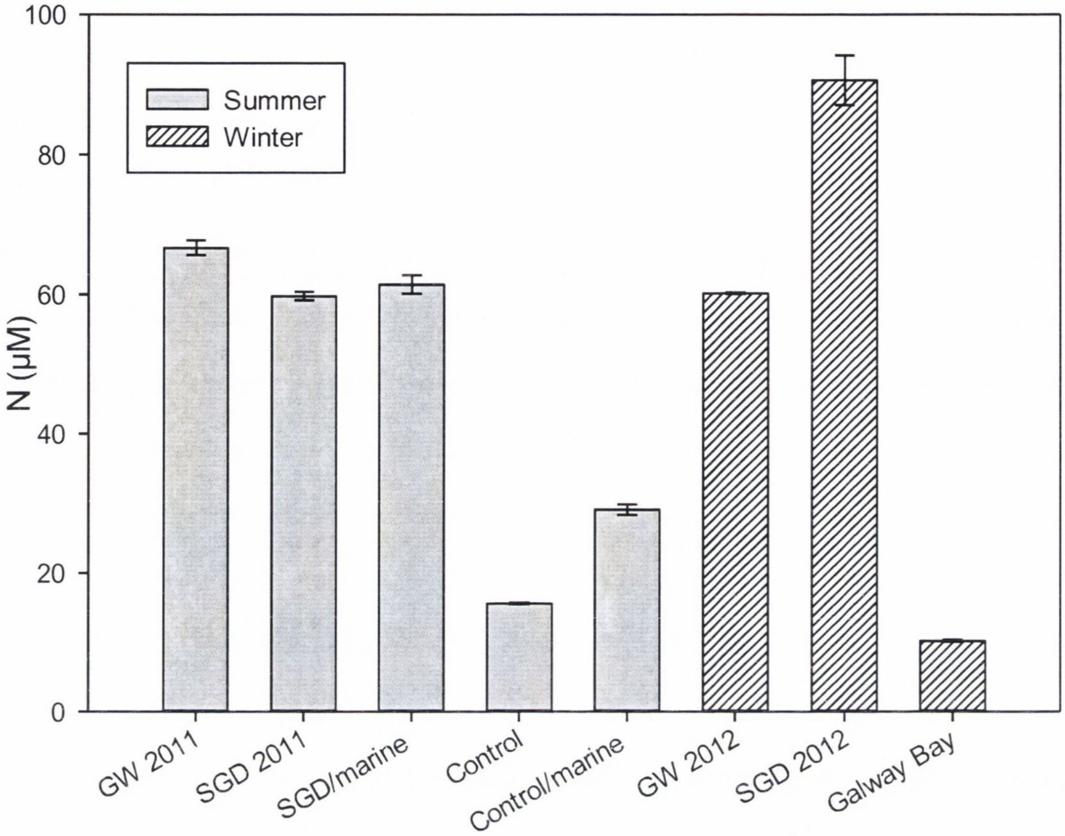


Figure 5-3 Summer and winter water nitrogen (nitrate and nitrite, µM) concentrations. *GW* indicates the groundwater sample (*n* = 4 in summer, *n* = 3 in winter). *SGD* is SGD site 1 (*n* = 3 in summer and *n* = 9 in winter). *Control* is control site 2 in summer (*n* = 5). *SGD/marine* and *control/marine* are the marine areas associated with the SGD and control site in summer (*n* = 3 for SGD/marine and *n* = 4 for control/marine), and *n* = 3 for the *Galway bay* sample in winter.

In summer, the nitrogen concentration of SGD, groundwater and the SGD/marine site was similar, but higher than either site 2 (control), or the control/marine site (Figure 5-4; Table 5-1). The concentration of nitrogen at the control/marine site was nearly twice that of site 2 (control), but half that of SGD. In winter, the concentration of nitrogen in SGD was elevated above that of groundwater and 9 times greater than that of Galway bay. The concentration of nitrogen in SGD was higher in winter than summer, while that of groundwater was similar between seasons (Figure 5-4; Table 5-1).

5.4.2. Isotope and elemental analysis results

5.4.2.1. Water summer isotopic composition

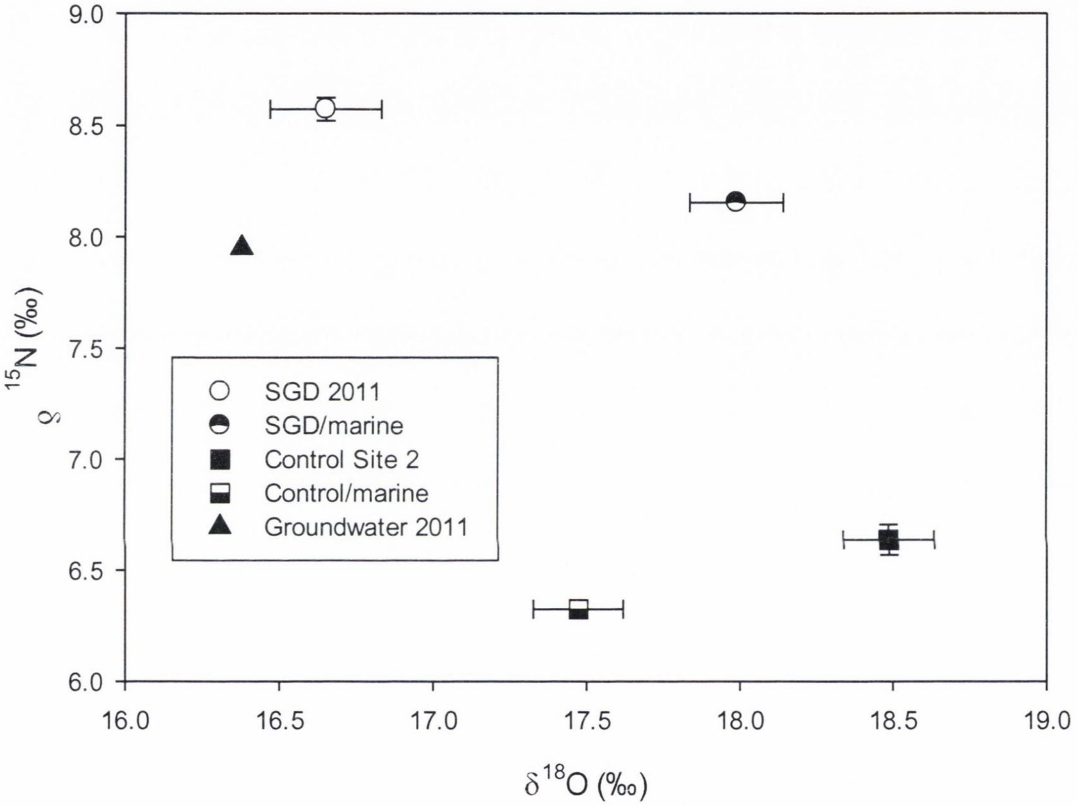


Figure 5-4 Scatter plot of mean (± 1SD, *n* = 2) δ^{15}N and δ^{18}O of nitrate in water in summer, showing SGD, control site 2, SGD/marine and SGD/control; and groundwater sample (*n* = 1).

SGD had the highest $\delta^{15}N_{NO3-}$ signature, followed by SGD/marine and groundwater (Figure 5-5; Table 5-1). While the $\delta^{15}N_{NO3-}$ of groundwater and marine water were both ~8 ‰, that of SGD was 0.5 ‰ higher, at 8.5 ‰. The $\delta^{15}N_{NO3}^{-}$ signature of the control site 2 and control/marine samples were similar, but ~2 ‰ lower than that of SGD (Figure 5-5; Table 5-1). The $\delta^{18}O_{NO3}^{-}$ of the groundwater sample and SGD were similar, while that of the two marine control area samples (SGD/marine and control/marine) were similar but higher than that of both the groundwater and SGD. The site 2 (control) sample had the highest $\delta^{18}O_{NO3^-}$ signature at ~18.5 ‰, which as ~2 ‰ higher than of the SGD and groundwater samples.

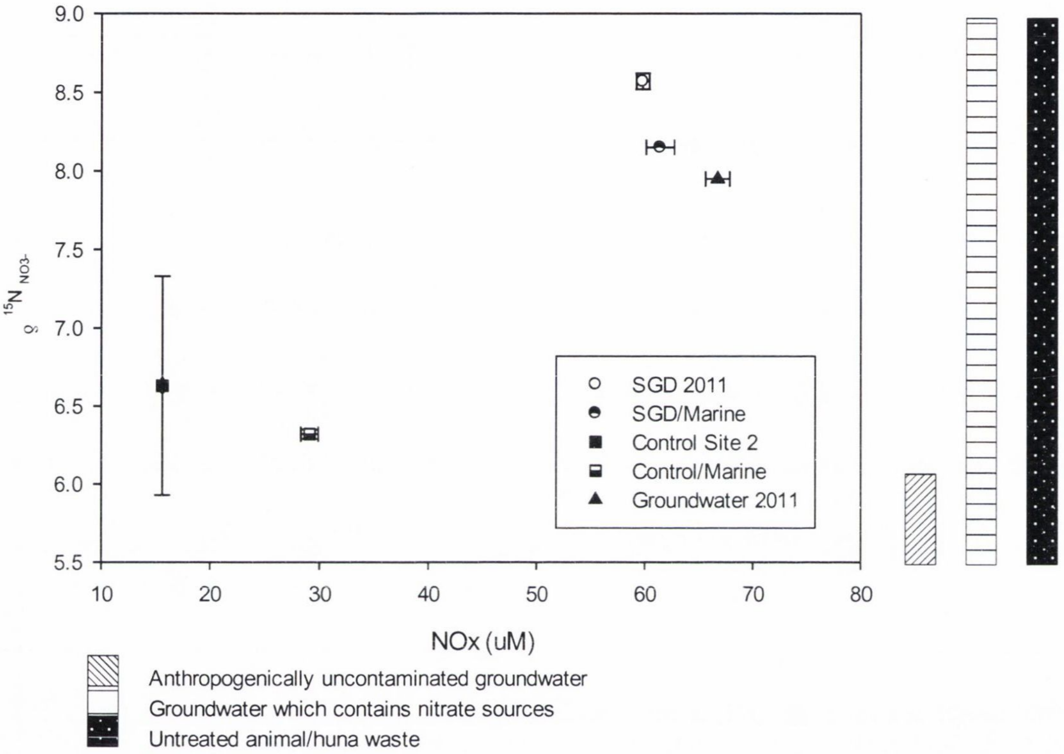


Figure 5-5 Scatter plot of NO_x (NO_2^- and NO_3^-) (± 1SE) versus $NO_3^-{}_{\delta 15N}$ data from the summer water sampling campaign. Reference literature isotopic values are indicated for anthropogencially uncontaminated groundwater (Xue et al. 2009), groundwater which contains nitrate sources (Xue et al. 2009), and the only groundwater nitrate source which overlaps this range, untreated animal/human waste (Mutchler et al. 2007).

The groundwater and two SGD compartments (SGD 2011 and SGD/marine) had elevated nitrogen concentrations and ^{15}N enriched nitrate, while the marine compartments (control site 2 and control/marine) had lower nitrate concentrations and ^{15}N depleted nitrate (Figure 5-5). Thus, increased nitrate concentrations in the groundwater and SGD compartments were associated with ^{15}N enriched nitrate. This is not a simple linear relationship however. Of the three high nitrate concentration compartments, groundwater had the highest nitrate concentration but also the most ^{15}N depleted nitrate. Conversely, of the three high nitrate concentration compartments, SGD had the lowest nitrate concentration but also the most ^{15}N enriched nitrate.

5.4.2.2. Macroalgal and macroinvertebrate isotopic results

5.4.2.2.1. Macroalgal statistical analysis

Isotopic results for algal samples collected per site were pooled for statistical analyses regardless of species. It is consistently documented in the literature that the isotopic compositions of algae of different species from the same site do not differ (Viana and Bode 2013; Derse et al. 2007; Umezawa et al. 2002).

Upon inspection of summer algal δ^{15}N data, there appeared to be two possible outliers in the dataset for each site (i.e. four outliers in total). All four data points were within ±3SD of the mean and data complied relatively well to the normal distribution (Anderson Darling test: p = 0.01 for both SGD site and control site algal δ^{15}N) with inclusion of these data points. Therefore, the data points were considered reasonable samples of the population and not removed as outliers. The variance of summer algal δ^{15}N data, however, was heteroscedastic (Levene's test for homogeneity of variance; p = 0.024 for δ^{15}N). To accommodate this, a Welch's T-test was used to compare the summer algal SGD site and control site δ^{15}N datasets. Welch's T-test doesn't assume that data have the same distribution and therefore has fewer degrees of freedom, but is more robust than Students T-test. Where data nearly conform to the assumptions of normality but violate the assumption of homoscedasticity, Welch's T-test is superior to the non-parametric equivalent Wilcoxon rank sum (Ruxton 2006). For summer algal

$\delta^{13}C$ data, the distribution of the control site data was non-normal (Anderson Darling test: $p = 0.001$), thus a non-parametric Wilcoxon rank sum test was used to compare these data.

5.4.2.2.2. Macroalgal isotopic results

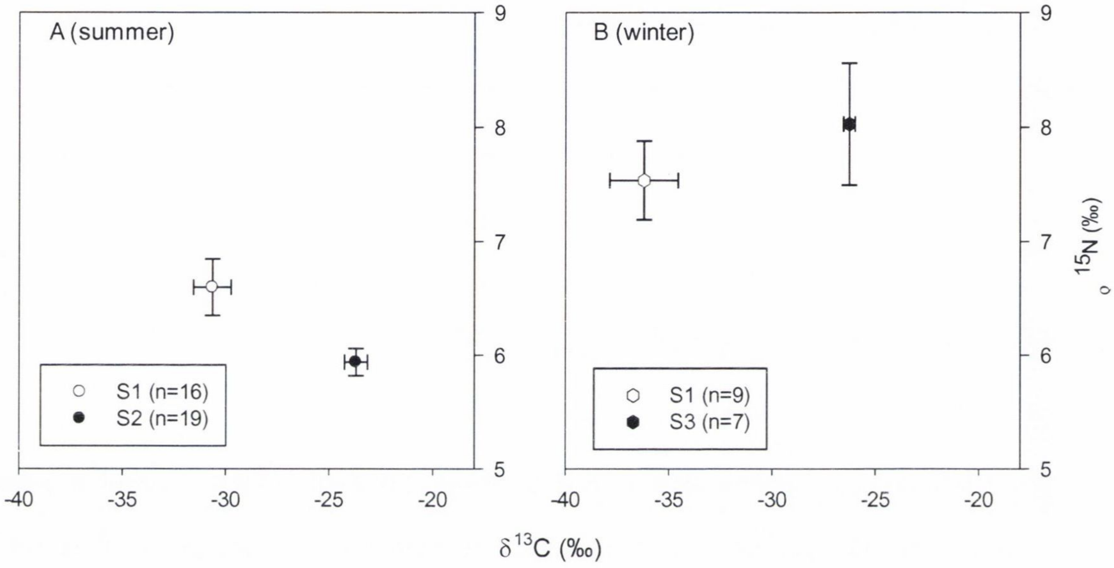


Figure 5-6 Scatter plot of algal mean $\delta^{15}N$ and $\delta^{13}C$ values (± 1SE) of samples from SGD site 1 (S1) and control site 2 (S2) in summer (A), and site 1 (S1) and control site 3 (S3) in winter (B).

Table 5-2 Results of Welch's T-test (T) and Wilcoxon rank sum test (W) comparing macroalgal and macroinvertebrate $\delta^{15}N$ and $\delta^{13}C$ values between SGD and control sites in summer and winter. In summer, $n = 16$ for site 1 and $n = 19$ for site 2 for macroalgae; $n = 3$ for both site 1 and site 2 for macroinvertebrates. In winter, $n = 9$ for site 1 and $n = 7$ for site 3 for macroalgae, and $n = 3$ for site 1 and $n = 9$ for site 3 for macroinvertebrates. *indicates significance ($\alpha = 0.05$ %).

Season	Sample	Test	$\delta^{15}N$	Test	$\delta^{13}C$
Summer	Macroalgae	T	0.037*	W	<0.000*
	Macroinvertebrate	W	0.1	W	0.076
Winter	Macroalgae	W	0.68	W	<0.000*
	Macroinvertebrate	W	0.009*	W	0.002*

Relative to site 2, site 1 algae were enriched in ^{15}N by ~0.5 ‰ in summer ($p = 0.037$) (Figure 5-7 (A); Table 5-2). In summer, the $\delta^{13}C$ value of algae sampled at site 1 was lower than that of the algae found at site 2, by ~8 ‰ (Figure 5-7 (A); Table 5-2; $p < 0.000$). In winter, for macroalgae at SGD site 1 and control site 3 there was no difference in mean $\delta^{15}N$ value, however the algal $\delta^{13}C$ value was lower at site 1 by ~10 ‰ (Figure 5-7 (B); Table 5-2; $p < 0.000$). The $\delta^{13}C$ value of macroalgae was lower in winter than summer at site 1. Macroalgal tissue was more ^{15}N enriched in winter than summer at site 1 and at the control sites, though the latter are not directly comparable as they were different sites.

5.4.2.2.3. Macroinvertebrate statistical analysis

Four invertebrate species were subject to $\delta^{15}N$ and $\delta^{13}C$ analysis. All species were either primarily detritivorous (supplemented with herbivory) or herbivorous (supplemented by detritivory) and belonged to approximately the same trophic level. Thus, the isotopic results of all species found at any one site were pooled for statistical analysis to increase the degrees of freedom and power of the test statistic.

The most abundant macroinvertebrate species recorded at both sites in summer was *Jaera albifrons* (Leach, 1814*).* All individuals from a quadrat were pooled to provide one sample per quadrat ($n = 5$), however, for two quadrats at both sites (site 1 and 2) insufficient sample material (minimum 1.2 mg) was available to conduct isotopic analysis. This reduced the sample size to three ($n = 3$) for both sites in summer. In winter, the most abundant species at the SGD site was again *J. albifrons*, found in sufficient biomass for analysis in all five quadrats. *Chaetogammarus marinus* (Leach, 1815) was also found in high abundance and sufficient biomass for analysis in two quadrats at site 1. At control site 3, *J.* albifrons was absent and *Corophium volutator* (Pallas, 1766) was the most abundant species, found in sufficient biomass for analysis in four quadrats. Sufficient biomass of *C. marinus* for analysis was found in one quadrat at site 3.

5.4.2.2.4. Macroinvertebrate isotopic results

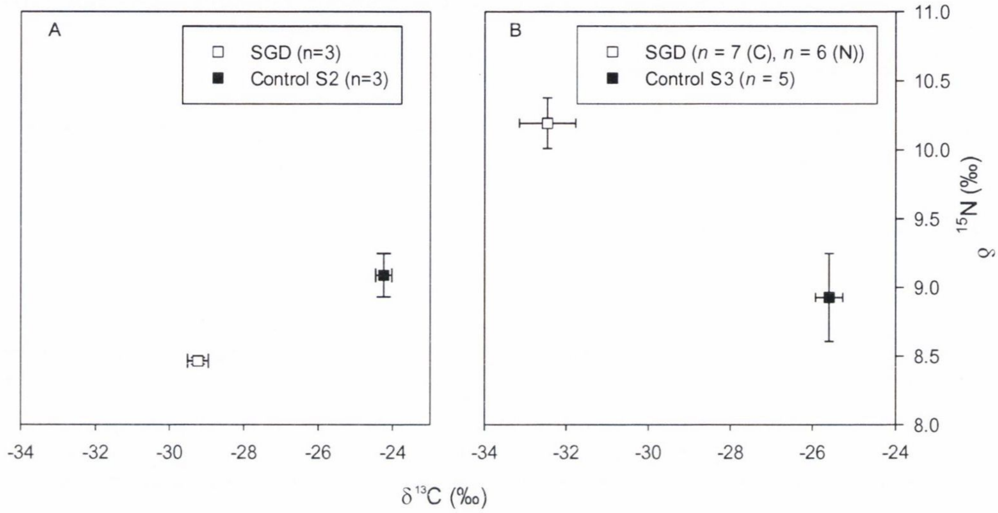


Figure 5-7 Scatter plot of mean macroinvertebrate (*J. albifrons*) δ^{15}N and δ^{13}C values (± 1SE) for samples from SGD site 1 (S1) and control site 2 (S2) in winter (A). Scatter plot of mean macroinvertebrate δ^{15}N and δ^{13}C values (± 1SE) of samples from SGD site 1 (*C. marinus* and *J. albifrons*) and control site 3 (S3) (*C. marinus* and *C. volutator*) in winter (B).

The δ^{13}C value of *J. albifrons* sampled in summer at site 1 was lower than that of site 2 by ~5.5 ‰, and the δ^{15}N value lower by ~0.5 ‰, though neither was statistically significant (Figure 5-8 (A); Table 5-2). The δ^{15}N value of macroinvertebrates from site 1 (*C. marinus* and *J. albifrons*, pooled) was ~2 ‰ higher (p = 0.009) and δ^{13}C value ~7 ‰ lower (p = 0.002) than site 3 macroinvertebrates (*C. marinus* and *C. volutator*, pooled) (Figure 5-8 (B); Table 5-2). Macroinvertebrate δ^{13}C values recorded at site 1 were lower in winter than summer.

5.4.2.2.5. Hypotheses

Null hypothesis (1) of no difference in the nitrogen isotopic signature of SGD food web components (macroalgae and macroinvertebrates) and their control food web counterparts cannot be rejected, as macroinvertebrates in summer and macroalgae in winter did not statistically differ between sites (Table 5-2). Null hypothesis (2), however, (that there is no difference in the carbon isotopic signature of SGD food web components (macroalgae and macroinvertebrates) and their control food web counterparts) is rejected.

5.4.2.3. Elemental analysis

Table 5-3 Elemental composition (C and N) and C:N molar ratio (mean and 1SE) of algal tissue collected at the SGD site 1 and control site 2.

	Site 1		Site 2			
	Mean	SE	Mean	SE		
n	16		19		*T*	*p*
%C	38.4	0.55	37.39	0.31	1.65	0.11
%N	2.58	0.23	2.52	0.17	0.21	0.84
C:N	18.66	1.03	18.52	1.05	0.1	0.92

Comparison using Welch's T-test (T) identified no difference in macroalgal %C, %N or C:N between the SGD site (site 1) and the control site (site 2).

Table 5-4 Elemental composition (C and N) and C:N molar ratio (mean and 1SE) of *Jaera albifrons* tissue collected at the SGD site 1 and control site 2.

	Site 1		Site 2			
	Mean	SE	Mean	SE		
n	3		3		*W*	*p*
%C	24.42	1.09	21.17	1.67	4	1
%N	5.2	0.24	5.2	0.34	8	0.2
C:N	5.48	0.08	4.73	0.06	9	0.1

Comparison using Wilcoxon rank sum test (*W*) identified no difference in macroinvertebrate (*Jaera albifrons*) %C, %N or C:N between the SGD site (site 1) and the control site (site 2).

5.5. Discussion

Relative to control and marine water samples, SGD nitrogen concentration was elevated and the pH depressed. The $\delta^{13}C$ values of macroalgae and macroinvertebrates from SGD site 1 were lower than that of the control site. These results indicate coastal nitrogen and carbon loading via karst-channeled SGD. In summer, the $\delta^{15}N$ value of SGD nitrate was elevated relative to control marine compartments, and this was mirrored in macroalgal $\delta^{15}N$ values. Macroalgal $\delta^{15}N$ and $\delta^{13}C$ values differed between the SGD and control sites in both seasons, though this was not significant for $\delta^{15}N$ values in winter. Macroinvertebrate $\delta^{15}N$ and $\delta^{13}C$ values at the SGD and control site differed, though only in winter. Analysis of food web component $\delta^{15}N$ and $\delta^{13}C$ values allowed discernment of SGD and control food webs; however, $\delta^{13}C$ values provided a more reliable indicator than $\delta^{15}N$ values in identifying and tracing pathways of N and C trophic transfer through SGD and control food webs.

The concentration of nitrogen in SGD was elevated relative to both Galway bay, and the within bay control sites. In summer, the nitrogen concentration of the control/marine was intermediate between that of site 2 (control) and SGD. This was likely due to mixing of the SGD with Kinvara bay water. The concentration of N at the SGD/marine site was comparable to that of SGD and the salinity of both samples was 0 psu. This indicates that the SGD/marine sample comprised freshwater SGD and dilution with bay water had not occurred (though N is not a conservative tracer of mixing).

Analysis by the Environmental Protection Agency (EPA) of groundwater sampled from the same location as the current study from 2003 to 2010, and SGD sampled from the same location as the current study site from 2007 to 2010 found a mean groundwater nitrate concentration of 78 µM ± 31 µM, with range 6 to 136 µM (Craig et al. 2010), while the mean nitrate level in SGD was 136 ± 62 µM, and ranging from 9 to 257 µM (O'Boyle et al. 2010). These results are congruent with those of the current study. Similarly, another study of karst-channeled SGD recorded the highest nitrate concentration (74 ± 11 µM) in groundwater that subsequently emerged as SGD a few meters off shore (exact distance offshore is not stated), while that of open bay water was 3 ± 5 µM (Mutchler et al. 2007). A previous study in Kinvara bay found SGD nitrogen concentrations which were significantly elevated above those found in the current study, though within the range report by O'Boyle et al. (2010), at ~230 µM in both winter 2005 and autumn 2006 (Cave and Henry 2011). In a study of eight karst springs in the region, Kilroy and Coxon (2005) found P concentrations increased following the first autumn rains due to release from the soil of phosphorus which had accumulated during the summer months due to fertilizer and manure application. It is likely that nitrate behaves in a similar way, though less pronounced due to increased solubility compared to phosphate, possibly explaining the higher winter than summer SGD concentrations in this study, and also possibly accounting in part for the elevated autumn/winter concentrations documented by Cave and Henry (2011).

The pH of marine water is typically ~8 and that of estuarine water 7.5 - 8.5 (Fogel et al. 1992). In the current study, the pH of water from both Galway Bay and control site 2 was ~8, in line with expectations. The pH of SGD was consistently ~7. The Environmental Protection Agency (EPA) measured pH and alkalinity of SGD from the period 2007 to 2012, 3 and 5 times per year. The average pH of the SGD over this period was 7.2, with standard deviation 0.26, and range from 6.8 to 7.9 (EPA, Anthony Mannix, pers. comm. 2014) consistent with the value recorded in the current study. The EPA found that the alkalinity of SGD ranged from 180 to 326 mg $CaCO_3$ with average 272.8 mg $CaCO_3$ and standard deviation 45 mg $CaCO_3$ (EPA, Anthony Mannix, pers. comm. 2014). The pH of water is determined by a number of factors, including the concentrations and speciation of dissolved inorganic carbon. Aquatic primary producers utilize CO_2 rather than HCO_3^- (Fogel et al. 1992) or CO_3^{2-}, as CO_2 moves freely across the cell membrane via diffusion while other forms of DIC require transport mechanisms or conversion to CO_2 prior to diffusion (Sharkey and Berry 1985). Thus, the distribution of inorganic carbon species influences carbon availability and limitation during primary production (Fogel et al. 1992). The relative concentration of CO_2 with respect to CO_3^{2-} and HCO_3^- is appreciably higher in freshwater (psu 0) than in seawater (psu 35) (Zeebe and Wolf-Gladrow 2001, p.9), i.e. there is a greater amount of C in its most biologically available form (i.e. as CO_2) in fresh than marine water. SGD can supply water column dissolved inorganic carbon (DIC) in coastal areas, and particularly carbonate coastal areas. Dorsett et al. (2011) estimated that 7 – 11 % of global coastal water DIC may be derived from SGD associated with karst and other carbonate systems. It is probable that SGD entering Kinvara bay associated C loading (in its biologically available form) and enhanced availability of C to primary producers.

The $\delta^{15}N_{NO3-}$ values of groundwater, SGD and SGD/marine water were all comparable and elevated by ~2 ‰ compared to site 2 (control) and control/marine samples. The $\delta^{15}N_{NO3-}$ value of SGD was within the range for untreated human/animal waste, while that of control samples was slightly (~0.05 ‰) above the range reported in the literature with reference to anthropogenically unpolluted surface waters. The elevated nitrogen concentration and enrichment in ^{15}N of groundwater and SGD is in line with expectations following contamination with a septic tank/animal waste. This is in good agreement with the known anthropogenic land uses in the region (i.e. primarily arable agriculture and tourism). The further ^{15}N enrichment of SGD nitrate relative to groundwater may indicate either (A) further additions of a ^{15}N enriched nitrogen source, and/ or (B) N fractionation due to processing during transit through the aquifer. As the concentration of nitrogen in SGD was less than that of groundwater, suggestion (A) is rejected. The lower nitrogen concentration and nitrate ^{15}N enrichment of SGD relative to groundwater (by ~6 µM and ~0.5 ‰, respectively) instead suggest the occurrence of a fractionating nitrogen removal process during transit between the groundwater borehole and site of SGD discharge (~1 km linear distance), i.e. suggestion (B). The most common nitrate removal process in groundwater is denitrification (Slomp and Van Cappellen 2004). As this microbially driven process requires anoxia to proceed, its occurrence is largely ruled out in the case of karst aquifers as these are normally well oxygenated (Slomp and Van Cappellen 2004). Though anoxia is a general requirement for denitrification, it has been documented that denitrification may occur within anoxic microsites or 'pockets' in an otherwise oxygenated water body. Jahagnir et al. (2013) found that denitrification occurred at a range of dissolved oxygen concentrations in two Irish aquifers which were composed of sand, gravel and karstified limestone and had high permeability and high dissolved oxygen concentration. Jahagnir et al. (2013) used concentrations of dissolved gases to suggest that denitrification occurred in anaerobic microsites in these aquifers. This mechanism is not uncommon across different systems- denitrification in anaerobic microsites in otherwise aerobic environments has been reported for soils (Koba et al. 1997), coastal marine sediments (Brandes and Devol 1997) and groundwater (Seiler and Vomberg 2005). Denitrification in aerobic groundwater occurs in anaerobic microsites formed by particulate organic matter (Jacinthe et al 1998; Hamersley and Howes 2002) and biofilms (Seiler and Vomberg 2005). Thus it is plausible that denitrification may occur in anaerobic microsites in the current study system, reducing the nitrate concentration of

SGD relative to groundwater and resulting in the observed ^{15}N enrichment in nitrate in SGD relative to nitrate in groundwater.

The $\delta^{18}O_{NO3-}$ of groundwater and SGD samples were similar and depleted in ^{18}O by 1 - 2 ‰ compared to all other samples. The $\delta^{18}O_{NO3-}$ value of water sampled at site 2 (control), and the control/marine and SGD/marine sites, was within the range expected for marine nitrate, while that of groundwater and SGD was depressed reflecting the fresh groundwater oxygen source (Figure 5-9). The $\delta^{18}O_{NO3-}$ value of SGD was slightly elevated above that expected for nitrate from human/animal waste. This increase in the nitrate δ^{18}O value may have been caused by H_2O isotopic enrichment due to evaporation, a bacterial nitrification process which utilizes a greater amount of atmospheric O_2 in the low pH environment (Xue et al. 2009), or a contribution from synthetic nitrate fertilizer.

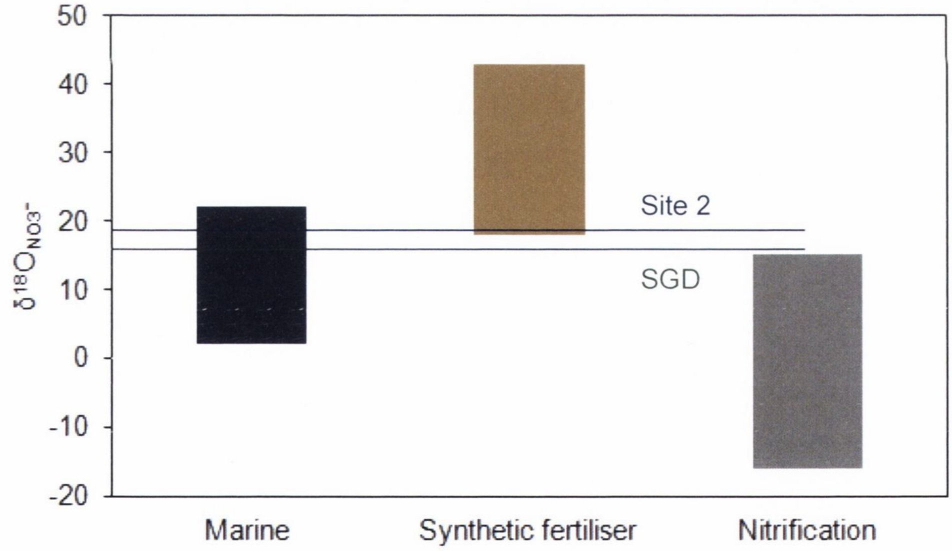


Figure 5-8 Literature reported ranges of $\delta^{18}O_{NO3-}$ values of marine water, synthetic fertiliser, and nitrate produced from nitrification of animal and human waste, adapted from Xue et al. (2009) and Kendall et al. (2007, p.381). The values for SGD and control site 2 reported herein are indicated with lines.

Macroalgal $\delta^{15}N$ values confirmed that the nitrogen source was enriched in ^{15}N in summer; however the difference in algal $\delta^{15}N$ values between the SGD and control site was less pronounced (0.7 ‰), and the $\delta^{15}N$ value of algae from site 1 was 2 ‰ lower than that of SGD nitrate. This difference in isotopic signature may be explained by temporal differences associated with the two analysis types. Algal $\delta^{15}N$ values represent the integration of all available nitrogen sources over time scales of days to weeks (Dailer et al. 2010) while a water sample is snapshot in time of what might be a temporally variable parameter. The temporal variability associated with water sampling is integrated into the algal isotopic signature. Also, where nitrogen is in excess, i.e. supply exceeds demand, isotopic fractionation may occur (Marshall et al. 2007, p.36; Mariotti et al. 1981) during the metabolic reduction of nitrate by the nitrate reductase loop prior to assimilation; previous studies have reported fractionation of up to 4 - 5 ‰ (Kendall et al. 2007, p.393). Thus, the lower algal $\delta^{15}N$ value relative to the SGD $\delta^{15}N_{NO3-}$ value may be the result of isotopic discrimination associated with SGD nitrogen loading. Finally, SGD site algae may derive some of their N requirement from ^{15}N depleted Kinvara bay water upon tidal influx, depressing the algal $\delta^{15}N$ value relative to that the SGD nitrate source $\delta^{15}N$ value. All measured algal $\delta^{15}N$ values fall within the range of global measures of algae from wastewater areas, + 4 to + 25 ‰ (Dailer et al. 2010) but were elevated compared to values reported for marine environments which are not contaminated with anthropogenic nitrogen, normally ~+ 3 to + 4 ‰ (Costanzo et al. 2001), supporting the suggestion of a wastewater/septic tank effluent nitrogen source in SGD.

Macroalgae were more ^{15}N enriched in winter than summer at all sites, though the control sites are not directly comparable as different sites were sampled each season. The isotopic signature of macroalgae is conditioned primarily by three factors, (1) the isotopic signature of the nitrogen source; (2) the extent of processing (normally nitrification and denitrification) of the nitrogen before assimilation by the algae as this may alter (normally elevate) the $\delta^{15}N$; and (3) the concentration of nitrogen available to macroalgae for assimilation. As previously introduced, different nitrogen sources have different isotopic signatures (Xue et al. 2009). The isotopic signature of nitrate in groundwater and SGD in summer indicates a largely human/animal wastewater groundwater nitrogen source. If algae tissue directly reflected the isotopic signature of its N source, we would expect to also find a similar isotopic signature in the algae (i.e. ~5 - 9 % (Mutchler et al. 2007)). In both seasons, the isotopic signature of algae at the SGD site was within this range, however, algal tissue in winter was ^{15}N enriched relative to algal tissue in summer.

The difference in isotopic signature between the seasons may be due to differences in nitrogen sources, enhanced by a difference in the concentrations of nitrogen present in SGD. The N isotopic signature of primary producers reflect that of the N nutrient source with fractionation proportional to the excess of nutrient supply relative to demand (Marshall et al. 2007, p.36; Mariotti et al. 1981; Kendall et al. 2007, p.393; Raven et al. 2002; Maberly et al. 1992). There is greater anthropogenic pressure from farming and tourism in summer. This derives from the application of synthetic and organic fertilizer, increased contamination with animal waste from animals on pasture which are kept in sheds over winter, and increased human waste associated with increased human population due to summer tourism. However, there is also greater N uptake by terrestrial vegetation in summer compared to winter. Furthermore, there is less evapotranspiration and thus greater aquifer recharge in winter relative to summer. Well yield data indicate a high degree of variability of the aquifer properties and flow (GSI 2004). Water table levels have high annual variations, which indicates that the storativity is low (Daly, 1985). Springs in the area (including the SGD spring) reflect the low storativity as many of the spring flows rise and fall quickly in response to rainfall events. Thus increased recharge in winter will rapidly flow toward the coastal springs, perhaps 'washing out' the nutrients which have accumulated in the soils and vasdose zone during the spring, summer and autumn has been described for P in eight karst aquifers elsewhere in the region (Kilroy and Coxon 2005). The discharge rate of SGD in Kinvara bay has been estimated at 9.75 – 10 m^3/s which discharge higher in winter than in summer (McCormick et al. 2014).

These processes result in greater concentrations of nitrate in groundwater and SGD in winter than summer. This was found in the current study for SGD, and by the EPA operational groundwater monitoring data from 2003 to 2010, which show that winter groundwater nitrate concentrations in the karst aquifer in the study site are higher than summer (98 ± 26 µM vs. 56.5 ± 33.6 µM (mean ± 1SD)) (Anthony Mannix, pers. comm. 2014). The reduced concentration of nitrate in summer relative to winter may result in less apparent fractionation between the SGD nitrate pool and macroalgae. The algal ^{15}N depletion in summer may indicate a greater contribution of N from synthetic fertilizer nitrogen (which is ^{15}N depleted). The influence of this source decreases as the growing season continues and human/animal waste become the main source of nitrogen, resulting in the observed algal ^{15}N enrichment in winter. This illustrates one of the benefits of using SIA of organisms rather than a 'snapshot in time' water sample to interpret environmental conditions. The water sample reflects the isotopic signature of nitrate in water on one day (or a restricted, normally temporarily stochastic, subset of days) on the year and is subject to greater variation relative to the algal sample which reflects nitrogen characteristics integrated over a number of weeks. The water sample indicates a largely animal/human waste nitrogen source in summer, while the macroalgal tissue indicates some contribution of a depleted ^{15}N source, most likely synthetic N fertiliser.

The mean δ^{15}N value of macroinvertebrates at the SGD site was 2 ‰ higher than that of the control site in winter. The δ^{15}N value of an organism reflects that of its food stuff with an increase of ~+ 2.5 to + 3.4 ‰ per trophic level (DeNiro and Epstein 1981; Zanden and Rasmussen 2001). Consumers integrate their nutrient sources over more extended time periods than primary producers and hence their isotopic signatures are subject to less short-term variability. *J. albifrons*, found at the SGD site in both years and the control site in summer, feeds by scouring and abrading algal or bacterial films from rocks or *Fucus* (Jones 1972). *C. marinus*, found at the SGD and control site in winter, is mainly a herbivore though as a Gammarid it may have some plasticity in terms of feeding type and may predate on other macroinvertebrate species (Macneil et al. 1997). *C. volutator*, the most abundant species at control site 3, is a suspensivore which predates primarily on plankton but which can switch to deposit feeding at low plankton concentrations (Møller and Riisgard 2006). Intuitively, it might be assumed that the winter sampling event may have coincided with low plankton concentrations and thus possibly a shift to deposit feeding mode in *C. volutator*. The data however contradict this as the mean (± SD, $n = 4$) δ^{15}N value of *C. volutator* was 8.94 (± 0.8) ‰, and the δ^{15}N value of the mainly herbivorous *C. marinus* from the same site was 8.88 ‰. Thus, the data indicate that *C. volutator* and *C. marinus* belong to the same trophic level. Though both *C. marinus* and *C. volutator* may display some plasticity in their feeding mode, they are both primarily herbivorous (Macneil et al. 1997; Møller and Riisgard 2006). It is acknowledged that error may be introduced by (1) plasticity in feeding habit away from herbivory for both species, and (2) reliance on only one sample, which may not be an accurate reflection of the true population for *C. marinus*.

However, to allow for comparisons to be made herein, it is assumed that both species belonged largely to the herbivore trophic level.

Based on macroinvertebrate values and known trophic enrichment factors, in winter, primary producer δ^{15}N values were expected to range from + 7.6 to + 8.5 ‰ at the SGD site and + 5.6 to + 6.5 ‰ at the control site. The mean δ^{15}N value of macroalgae at the SGD site was within the expected range, at + 8 ‰, however, the control site macroalgal mean δ^{15}N value was outside the expected range, at + 7.5 ‰. Also, contrary to expectation, the two sites overlapped in their ranges of macroalgal δ^{15}N values (SGD site δ^{15}N values: + 7.2 to + 7.9 ‰ and control site δ^{15}N values: + 7.5 to + 8.6 ‰). These anomalies may reflect short term deviations and similarity in the isotopic signature of the nitrogen source entering the food web. Winter macroinvertebrate δ^{15}N values indicate, however, that over a more extended time frame, the nitrogen entering the SGD food webs was enriched in ^{15}N relative to the control site, by ~2 ‰, similar to that of nitrate in SGD in summer.

Literature reported values for nitrogen isotopic signature of *C. marinus* vary widely, ranging from + 4.6 to + 18 ‰, while δ^{13}C values range from - 19 to - 16.2 ‰ (Schaal et al. 2010; Van Ael et al. 2013). A similar pattern is found in the literature for *C. volutator* with δ^{15}N values ranging from + 7.4 to + 20.3 ‰, and δ^{13}C values from - 18.2 to - 15.2 ‰ (Riera et al. 2004; Creach et al. 1997). No data is available on the isotopic signature of *J. albifrons*. Isotopic analysis of the closely related *J. istri* found δ^{15}N values ranging from + 10.03 to + 14.74 ‰ and δ^{13}C values from - 27.22 to - 26.22 ‰ (Van Riel et al. 2006). The δ^{15}N values of macroinvertebrate species in the current study are close to those reported in the literature. As macroinvertebrate δ^{15}N and δ^{13}C values are conditioned largely (though not exclusively) by the isotopic signature of the N and C sources rather than particulars of the species, a large degree of variation is expected between studies, as observed, particularly for $\delta^{15}N$. With this in mind, it is noteworthy that the $\delta^{13}C$ values recorded for all three species in the current study are below any previously reported range, particularly for *C. marinus* and *C. volutator*.

Algal $\delta^{13}C$ values were lower at the SGD site relative to control sites in both years. Control site algae $\delta^{13}C$ values were within the ranges reported for estuarine macroalgae (- 8 to - 27 ‰ (Michener and Kaufman 2007, p.256)). Values from the SGD site were however outside the current documented minimum ranges for their genera and among some of the lowest recorded in the literature (Table 5-5). This may be explained in part by the freshwater nature of the DIC source (i.e. SGD) as freshwater DIC is depleted in ^{13}C relative to seawater and estuarine algae are generally depleted in ^{13}C ($\delta^{13}C$ values as low as - 27 due to utilisation of a freshwater DIC source (Michener and Kaufman 2007, p.256). Previously, only 25 marine algal species have been recorded with $\delta^{13}C$ vales less than - 30 ‰, three Chlorophytes and 22 Rhodophytes (Raven et al. 1995; Raven et al. 2002). Never before have values as low as those recorded from the SGD site (- 32 ‰ in summer, and - 36 ‰ in winter) been observed for a Fucoid or any member of the Phyaeophyta. Marine algae belonging to *Ulva* spp., *Fucus* spp. and other genera collected from the Atlantic generally range in $\delta^{13}C$ values from - 8 to - 22 ‰ (Bode et al. 2006). For example, the $\delta^{13}C$ values of *Ulva* spp. collected on intertidal rocky shores of Galicia, Spain, ranged from - 14.4 to - 15.1 ‰ and *Fucus* spp. from - 13.5 to - 15.3 ‰ (Bode et al. 2006).

Table 5-5 (A) Published ranges of algal $\delta^{13}C$ values for all marine macroalgae (Marine MA), estuarine macroalgae (Estuarine MA), and the three algal phyla. Mean, minimum (Min.) and maximum (Max.) values are reported in ‰. (B) Mean and SE of macroalgal $\delta^{13}C$ values documented in the current study, at each site in each season. Winter SGD (S1) value is highlighted in bold as it is below the range currently documented in the literature (Fry and Sherr 1989; Kerby and Raven 1985; Maberly et al. 1992; Raven et al. 2002; Vizzini and Mazzola 2003; Fredriksen 2003).

(A) Literature values	$\delta^{13}C$		
	Mean	Min.	Max.
Marine MA	–17	–35	–3
Estuarine MA		–27	–8
Chlorophyta	–16	–21.2	–8
Phaeophyta	–17	–20.8	–10
Rhodophyta	–23	–35	–11

(B) Study values		$\delta^{13}C$	
Season	Site	Mean	SE (n)
Summer	SGD (S1)	-30.8	0.8 (16)
	Control (S2)	-23.7	0.55 (19)
Winter	SGD (S1)	**-37.0**	1.6 (9)
	Control (S3)	-26.1	0.29 (7)

The very low algal $\delta^{13}C$ values at the SGD site may indicate CO_2 use only, as previously introduced. Reduced availability of CO_2 and the resultant increase fractionation of the CO_2 pool, as well as, possibly, some dependence on CCMs and HCO_3^- utilisation may explain the control sites' relatively higher $\delta^{13}C$ values (summer mean: - 24 ‰; winter mean: - 26 ‰).

Macroalgal $\delta^{13}C$ values were lower in winter than summer across all sites. Low $\delta^{13}C$ values of particulate organic matter, phytoplankton, algae and consumers in winter are widely documented (Vizzini and Mazzola 2003; Brenchley et al. 1997; Simenstad and Wissmar 1985; Conkright and Sackett 1986; Lehmann et al. 2004). Seasonal depletions of up to 8 ‰ have been recorded in algae and consumers in estuarine and near shore habitats (Simenstad and Wissmar 1985). For example, Brenchley et al. (1997) found the $\delta^{13}C$ value of *Fucus serratus*, an intertidal species common in the NE Atlantic, dropped from - 13.5 ‰ in summer to - 18 ‰ in winter. Lower algae $\delta^{13}C$ values in winter relative to summer in the current study were due to the seasonal decrease in CO_2 demand relative to supply.

The algal $\delta^{13}C$ values observed at the SGD site are however below the range explained by simple CO_2-only utilisation or seasonal differences. Algal $\delta^{13}C$ values are determined largely by [CO_2 (aq)] and are normally depleted in ^{13}C relative to their source (Marshall et al. 2008, p.22). Using this relationship between organic matter $\delta^{13}C$ values and [CO_2(aq)], the unusually low algal $\delta^{13}C$ values at the site may be explained by high [CO_2 (aq)] associated with SGD loading of CO_2. Based tentatively on the data of Lehmann et al. (2004) (Figure 5-2), a decrease in POM $\delta^{13}C$ value (analogous to algal $\delta^{13}C$ value here) by 10 ‰ (i.e. the difference between and control winter values), corresponds to a ~80 % (note, per cent) increase in surface freshwater [CO_2 (aq)], from 0.69 μg L^{-1} (i.e. ln(3)) to 1.25 μg L^{-1} (ln (3.5)). This theory is corroborated by other data which found that, theoretically, marine algal cells grown in large excess of total DIC should have $\delta^{13}C$ values of about - 36 ‰ (Fogel et al. 1992). Through further experimentation involving analysis of the $\delta^{13}C$ values of both DIC and organic carbon compartments, the $\delta^{13}C$ values of the latter may be used to infer the degree of C loading associated with SGD and thus the effect of SGD on the carbon cycling, trophic transfer, and coupling of terrestrial and oceanic carbon cycles.

SGD water temperature, pH and salinity remained constant between seasons, but were altered compared to the receiving marine water. Groundwater temperature in Ireland generally ranges from 9.5 – 11.0 °C (Aldwell and Burdon 1986), congruent with the results of the current study except in summer when groundwater temperature was 16.4 °C despite SGD water being within the expected range, at ~11 °C. During routine monitoring of the same groundwater borehole, the Environmental Protection Agency measured similar groundwater temperatures, at 18 °C in June and 16.9 °C in September 2010 (Anthony Mannix, pers. comm. 2014). Groundwater temperature analysis of this borehole by the Environmental Protection Agency from the years 2004 – 2012 found that the mean temperature was higher than reported average groundwater temperature, at 12.1 °C, with a relatively high associated standard deviation (2.6 °C), and groundwater temperature ranged from 8 to 16.9 °C (Anthony Mannix, pers. comm. 2014). This borehole is located in a permanent pumping station which services the surrounding hinterland with potable water. The borehole is approximately 70 m deep and the water accessed via a permanent pump. The high readings recorded for this borehole relative to known groundwater temperature were likely an artifact of the pumping station mechanics.

The temperature of Kinvara Bay water ranges from 17.92 °C in summer to 6.95 °C in winter (EPA, 2010); values congruent with those for marine samples in the current study. Summer SGD temperature was reduced and winter temperature elevated relative to the receiving marine water. Temperature influences the rate of metabolic reactions with increased temperature generally associated with increased reaction rates, and vice versa. This can be seen in non-nutrient limited estuaries where phytoplankton photosynthetic rates vary with season due to temperature, with higher rates in warmer seasons and lower rates in colder seasons (Eppley 1972). Reduced water temperature in winter relative to summer is associated with reduced metabolic and photosynthetic rates. Also, the solubility of CO_2 in water increases as water temperature decreases (Wiebe and Gaddy 1940). Thus, reduced SGD temperatures relative to surrounding water in summer, increases the supply of dissolved CO_2 relative to algal demand in two ways, by decreasing algal CO_2 incorporation and increasing water [CO_{2aq}]. Thus temperature regime may condition the relationship between algal CO_2 demand and water CO_2 supply and enhance algal ^{13}C depletion at the SGD site in summer and all sites in winter.

Macroinvertebrate $\delta^{13}C$ values closely mirrored macroalgal values across all sites with negligible increases (< 1 ‰). This allowed clear discrimination of higher tiers of food webs in receipt of SGD, and control food webs. Reduced $\delta^{13}C$ values of food web components are normally attributed to utilisation of terrestrial carbon sources. In some cases however, these patterns may instead result from food web utilisation of freshwater SGD-borne DIC fluxes. This has implications for studies of coastal food web C use and transfer in systems in receipt of freshwater SGD, and karst-channeled SGD, in particular.

Tissue nutrient content (%C and %N) for macroalgae and *J. albifrons* did not differ between the SGD site and control site 2. Macroalgal and *J. albifrons* C:N ratios did not differ statistically between SGD site 1 and control site 2. This indicates that parameters which varied due to the presence of SGD, including salinity and nitrogen concentration, did not alter algal and *J. albifrons* uptake metabolism of N and C at the individual sites. These results indicate the superior efficiency of stable isotope analysis relative to analysis of elemental data in the study of nutrient trohpic transfer associated with SGD in the current system.

Stable isotope analysis was employed as a suitable technique to (1) trace N and C trophic transfer, thus identifying two discrete food webs and pathways of nutrient trophic transfer, one associated with SGD and the transfer of SGD-borne C and N, and the other associated with the control environment and trophic transfer of control environment C and N; (2) indicate SGD carbon loading; and (3) tentatively indicate the source of SGD-borne N. Previously, Kamermans et al. (2002) found that freshwater SGD was associated with the seagrass (*Thalassodendron ciliatum*) enrichment in ^{15}N, with seagrass δ^{15}N values ranging from ~+ 2 ‰ in the absence of SGD to ~+ 6 ‰ at the most SGD site. These results corroborate the results found herein, i.e. the utility of SIA in delineating SGD and control food webs. However, Kamermans et al. (2002) considered one seagrass species only and the study was wholly reliant on differences in nitrogen isotopic signature, which, from the current study is a less reliable and consistent indicator than carbon isotopic signatures. Mutchler et al. (2007) studied the effect of wastewater conveyed via karst-channeled subtidal SGD on seagrass δ^{15}N values along coast of the Yucatan peninsula, Mexico. Though a positive relationship was observed between freshwater influence and water nitrate ^{15}N enrichment, contrary to the results of the current study, this was not mirrored in algal tissue (Mutchler et al. 2007). Sole reliance on nitrogen isotopic composition by Mutchler et al. (2007) hindered apportionment of a relationship between macroalgae and SGD. Similarly, Carruthers et al. (2005) who studied wastewater conveyed via SGD along a nearby portion of the same karst coastline of the Yucatan peninsula, analysed the δ^{15}N of a single species of seagrass (*Thalassia testudinum*), which provided somewhat ambiguous results. The δ^{15}N value of seagrass tissue sampled from close to the site of SGD was low (+ 1.9 ± 0.81 ‰) and similar to seagrass tissue at a distance from the site (+ 1.69 ± 0.88 ‰) (Carruthers et al. 2005). The lowest SGD salinity recorded was however 33.3, indicating that SGD comprised mostly marine water (Carruthers et al. 2005). From comparison with the current study in which carbon isotopic signatures were the more reliable tracer/indicator, focus on the nitrogen isotopic signature only prohibited Carruthers et al. (2005) from determining utilisation of SGD-borne nutrients by the selected seagrass species.

Further research analysed sediment organic matter (SOM), and macroalgal and macroinvertebrate δ^{13}C and δ^{15}N values in a study of freshwater SGD on a French intertidal sand flat (Ouisse et al. 2011). Similar to the current study, they found that freshwater SGD was associated with SOM ^{13}C depletion (~- 19 ‰ compared to control site ~- 15 ‰) and SOM ^{15}N enrichment (+ 11 ‰, compared to control site + 7.5 ‰). Unlike the current study, Ouisse et al. (2011) didn't compare algal isotopic signatures from control and SGD sites. Ouisse et al. (2011) ascribe the reduced SOM δ^{13}C values to a terrestrial origin of the intertidal sediment organic matter, transported by SGD. The results of the current study however, which measured δ^{13}C value of macroalgae (i.e. the primary producer source of organic matter) reduce the possibility of terrestrial origin of organic matter, though some contribution may have derived from remineralisation of terrestrial organic carbon. Ouisse et al. (2011) found that *Hydrobia ulvae* collected at the SGD site was more enriched in ^{12}C than control site *H. ulvae*. The current study confirms this pattern of macroinvertebrate ^{13}C depletions in connection with SGD. Thus, the results of the current research clarify the findings of previous studies, indicating that SIA may be a viable technique in SGD ecological research, however in terms of isotopic signatures; reliance on nitrogen isotopic signature alone may be insufficient.

For the same sample size the difference in the δ^{13}C value between the SGD and control site was larger than the difference in δ^{15}N for both macroalgae and macroinvertebrates. Thus, for all comparisons, there was greater statistical significance associated with the difference in δ^{13}C values than the difference in δ^{15}N values between SGD and control sites (Table 5-2). While three out of four comparisons were statistically significant for δ^{13}C, only one out of the four comparisons found a statistically significant difference for δ^{15}N (Table 5-2). Thus δ^{13}C values provided a more reliable indicator than δ^{15}N in identifying and tracing pathways of nutrient through SGD and control food web. For macroinvertebrates in summer, though difference in both $\delta^{13}C$ and $\delta^{15}N$ between the SGD and control were non-significant, the *p*-value obtained for the $\delta^{13}C$ comparison was lower than that obtained for the $\delta^{15}N$ comparison.

This research is subject to a number of experimental weaknesses. Firstly, the lack of statistical significance for macroinvertebrate $\delta^{15}N$ and $\delta^{13}C$ values in summer and macroalgal $\delta^{15}N$ values in winter may be due to the use of insufficient sample sizes (i.e., *n*). Though some of the sample sizes (*n*) used in the current study were small, they are however in line with those normally used under the constraints imposed when studying SGD, where sample sizes of only 1 ($n = 1$) occur, ~3 ($n = 3$) are usual and individual sample sizes of up to 5 infrequent (e.g. Ouisse et al. 2011 ($n = 1, 3, 5$); Carruthers et al. 2005 ($n = 5$); Kamermans et al. 2002 ($n = 2, 3, 5$)). The standard deviations found in the current study may be used to calculate the required sample size for a given significance level (α) and power to detect a difference or probability of a type 11 error (β) using Z values derived from the standard normal statistical table (Mullins 2010, pp.24–25). The formula to calculate the required sample size within the bounds of these parameters is given by

$$n = [(Z_\alpha + Z_\beta)^2 \, 2\sigma^2] / \delta^2$$

where

n = sample size

Z_α = the Z value given by the significance level, α

Z_β = the Z value that controls the type 11 error, β

σ = approximate standard deviation associated with the system

δ = minimum difference between population means which will be detected (Mullins 2010, pp.24–25).

Table 5-6 shows the standard deviation (*σ*) recorded for both macroalgae and macroinvertebrates, and the associated sample size used for each site in each season. The largest standard deviation recorded per isotope for each of macroalgae and macroinvertebrates across the two seasons will be used for the calculation and this estimate will be rounded up to the nearest 0.5 to provide the most robust estimate of the required sample sizes.

Table 5-6 Standard deviations and sample sizes used in the current study. LR is the value of the largest standard deviation, rounded up to the nearest 0.5. 'Macroinverts.' indicates macroinvertebrates.

		$\delta^{15}N$					$\delta^{13}C$				
		Summer		Winter			Summer		Winter		
	Site	S1	S2	S1	S3	LR	S1	S2	S1	S3	LR
Macroalgae	sd (σ)	0.9	0.5	1	0.8	1	3.2	2.4	5	0.8	3.5
	n	16	19	9	7		16	19	9	7	
Macroinverts.	sd (σ)	0.04	0.3	0.45	0.7	1	0.5	0.4	1.8	0.8	2
	n	3	3	**6**	5		3	3	**7**	5	

The observed differences in macroalgae and macroinvertebrate $\delta^{15}N$ values between SGD and control site samples were relatively small. For macroalgae, differences in the $\delta^{15}N$ value of 0.5 ‰ between SGD and control sites samples were found to be statistically significant. As one decreases the difference between the SGD and control site sample means which one would like to detect, there is a concomitant increase in the sample size (*n*) required in order to detect this difference. With this in mind, for comparative purposes, the sample sizes required to detect a difference of both 0.5 and 1 ‰ are calculated for $\delta^{15}N$. Differences in macroalgae and macroinvertebrates mean $\delta^{15}C$ values between SGD and control sites were relatively larger, in the order of 5 ‰

to 7 ‰. Thus, 5 ‰ and 7 ‰ are selected as the δ when calculating the required sample size for δ^{13}C. In the current study a significance level (α) of 5 % or 0.05 was applied across the board. From the standard normal table for a two tailed test, with $\alpha = 0.05$, Z_α is 1.96. Assuming a corresponding power of $1-\beta = 0.95$, or equivalently a Type 11 error probability of $\beta = 0.05$ is required, then $Z_\beta = 1.645$. The calculated sample sizes for the given desired detectable difference in population means are detailed in table 5-7. For full details of calculations, see Appendix F. For macroalgae and macroinvertebrate δ^{15}N values, the increase of 0.5 ‰ in detectable population mean difference (from 0.5 ‰ to 1 ‰) is associated with a large increase in sample size, jumping from 26 samples to detect a 1 ‰ difference in sample means to 104 samples to detect a 0.5 ‰ difference in population means. For δ^{13}C values, the calculated required sample sizes were relatively low across the board, ranging from only 2 ($n = 2$) for macroinvertebrates where $\delta = 7$, to 13 ($n = 13$) for macroalgae where $\delta = 5$. In the current study, the sample sizes used in some instances fall below the calculated required sample size, particularly for nitrogen isotopic analysis. Future experiments in the current system should endeavor to increase the sample sizes used for carbon isotopic analysis to $n = 13$ for macroalgae and $n = 4$ for macroinvertebrates, and for nitrogen isotopic analysis to at least $n = 26$ for both macroinvertebrates and macroalgae.

Table 5-7 Calculated sample sizes using sample size formula, also showing associated standard deviation as measured in the current study and detectable difference as approximated from the current study, for 1. Macroalgae and 2. Macroinvertebrates.

1. Macroalgae					
δ^{15}N (‰)			δ^{13}C (‰)		
δ	σ	n	δ	σ	n
0.5	1	104	**5**	4	13

1. Macroalgae					
δ^{15}N (‰)			δ^{13}C (‰)		
δ	σ	n	δ	σ	n
1	1	26	**7**	4	7

2. Macroinvertebrates					
δ^{15}N (‰)			δ^{13}C (‰)		
δ	σ	n	δ	σ	n

2. Macroinvertebrates					
δ^{15}N (‰)			δ^{13}C (‰)		
δ	σ	n	δ	σ	n

0.5	1	104	**5**	2	4	**1**	1	26	**7**	2	2

Due to logistical constraints, the bulk isotopic samples from each season were analysed in different laboratories on different instruments, as per the materials and methods. This resulted in different analytical errors (higher in winter than summer) associated with the two analyses. To overcome this caveat, it was assumed that the results are comparable; however, this assumption should be taken into account when comparing the results from site 1 between seasons. Also, different macroinvertebrate species were used for site 1 and site 3 isotopic comparisons in winter. Though these species belonged to the same broad trophic level, it may be that some of the disparity in isotopic signatures between macroinvertebrates at the two sites was due to species effect rather than the effect of SGD. This caveat, which has previously occurred in SGD research involving the use of isotopes (Ouisse et al. 2011), is one of the issues inherent in the study of the ecology associated with SGD as the physicochemical properties of SGD alter the environment making it more suitable for some organisms while excluding others. This issue relates back to what is probably the most significant caveat is the study of the ecological effects of SGD the availability of a sufficient number of suitable and control sites from which to obtain sufficient sample sizes of organisms to enable meaningful and statistically robust comparisons when stable isotope analysis of organisms is employed.

Using SIA, it was determined that Karst-channeled intertidal freshwater SGD increased N and C availability to associated marine food webs. Also, the δ^{15}N values of SGD_{NO3-} indicated that SGD-borne nitrogen may derive from wastewater/septic tank effluent source(s). Both macroalgal and macroinvertebrate species at the SGD site exhibited ^{13}C depletion in both seasons, and ^{15}N enrichment congruent with the results of the nitrate in water isotopic analysis in summer, evidencing two distinct trophic pathways. Macroalgal and macroinvertebrate $\delta^{13}C$ values were more useful than $\delta^{15}N$ values in delineating SGD and control food webs. Thus, organism $\delta^{13}C$ values may be more useful than $\delta^{15}N$ values in the study of SGD where the $\delta^{15}N_{NO3-}$ values of SGD and non-SGD water are not significantly divergent. The previously well-documented winter depletions in ^{13}C of macroalgae and macroinvertebrate tissue were observed in the current study. However, SGD was associated with severe ^{13}C depletion in algae, which had until now only been observed in a very restricted number of genera, mostly within the Rhodophyta. It is suggested that the mechanism responsible for this extreme depletion is a pronounced form of the process responsible for the winter algal ^{13}C depletions, i.e. increased carbon availability relative to macroalgal demand. In non-SGD systems, this process arises from a seasonal effect only, while SGD sites experience increased water DIC concentrations due to both the winter effect and the SGD DIC flux. That is, the results herein suggest that the unusually ^{13}C depleted algal tissue in winter may be due to a glut of CO_2 availability associated with SGD carbon loading. As previously discussed, this feature may potentially be used to determine [DIC (aq)] and [CO_2 (aq)] resulting from SGD carbon loading (Lehmann et al. 2004). Also, generally, in intertidal and coastal research, depletion of ^{13}C in organism tissue is often attributed to utilisation of terrestrial organic carbon sources which are normally ^{13}C depleted, however, it is demonstrated herein that severe ^{13}C depletion may also arise from utilisation of carbon which was fixed by in the marine environment. This has implications for our understanding of coastal food web resource use, and cycling of carbon between terrestrial and marine environments.

SIA of organism tissue is a nearly unused method in SGD research, however, as illustrated by the current study, this technique holds huge potential to further our understanding of not only SGD, but also coastal ecosystem functioning. The study herein links the nitrogen found in SGD water to the nitrogen found in the associated food web, implicating SGD-borne nutrients of terrestrial, and possibly of anthropogenic origin, in the functioning of intertidal ecosystems. SIA may provide a method of identifying incipient eutrophication in SGD systems, enabling early intervention and remediation. The study herein updates the current state of the art regarding useful methodologies in the study of the ecological alterations associated with SGD by indicating that SIA of organism N and C can provide a useful technique which provides a means of tracing SGD-borne nutrients into associated food webs. Furthermore, the current study updates the current state of the art by indicating that δ^{13}C values provide a more reliable indicator than δ^{15}N values in identifying and tracing pathways of nutrient trophic transfer and reliance on δ^{15}N values alone may lead to ambiguous results.

Chapter 6. General discussion

6.1. The Ecological Effects of SGD

Table 6-1 Results summary table showing results of multivariate analysis and univariate comparisons of ecological data, SGD salinity range and minimum recorded values, and maximum SGD nitrogen concentration recorded. SGD salinity and nitrogen concentrations quoted for the SGD site in the Ria Formosa were sourced from Leote et al. (2008), and the salinity data for the control sites (salinity of lagoon water) was sourced from Ferreira (2003). 'M' indicates ANOSIM multivariate comparison, and 'E' and 'D' the result of univariate comparisons. 'E' indicates that SGD sites were elevated in given parameter relative to the control sites, and 'D' indicates that SGD sites had a reduced value for the given parameter relative to the control sites. 'Diversity' indicates the number of species recorded in total at a given site. 'nd' indicates no difference. *significance at α = 5%. $^{-}$indicates that result applies to only one of the two site comparisons.

	Macroalgae		Macroinvertebrates			% cover of sessile species (macroalgae and attached macroinvertebrates)		Salinity (psu)				Maximum Nitrogen conc.
								SGD site		Control		
Site	Species richness (*S*)	Biomass	Species richness (*S*)	Biomass	Abundance	Organism cover	Species richness	Minimum	Range	Minimum	Range	
Ria Formosa Lagoon (Chapter 2)	E,	E,M*	E^{*-}	E^{-},M	E*M*			-17	(17 - 36)	(35.3)	(35.5 - 36.9)	(185 µM)
Kinvara Bay (Chapter 3)	nd	E*,M*	D	E^{-}	E*,M*	E,M*	E	0	0	10.8	10.8 - 13.9	100 µM
Olhos de Água (Chapter 4)						D,M*	D	4	4.27 - 31.3	32.6	32.6 - 36.1	515

Four surveys were conducted involving three system types across two countries. In all three systems, SGD was associated with altered ecology. This took the form of modifications in the composition and biomass of the macroalgal and macroinvertebrate assemblage (Chapter 2 and 3) and sessile species composition (Chapter 3 and 4) (Table 6-1). Though SGD was associated with ecological alterations in all systems, the form of these alterations differed between systems, even when comparing two instances of karst-channelled SGD.

SGD was associated with increased macroalgal biomass in Ria Formosa lagoon (Chapter 2) and Kinvara Bay (Chapter 3) (Table 6-1), and increased diversity of macroalgal species in the Ria Formosa lagoon. Diffuse subtidal SGD has elsewhere similarly been linked to increased primary production (Miller and Ullman 2004; Waska and Kim 2010; Migné et al. 2011) and changes to primary producer species diversity (Kamermans et al. 2002), congruent with the results herein. In Olhos de Água (Chapter 4), *Ellisolandia elongata,* which is normally the most abundant algal species in such systems, was excluded from the SGD rock pools and a negative correlation was found between the cover of *E. elongata* and the fraction of freshwater SGD in the relative control rock pools. Similar results showing a negative relationship between subtidal SGD inputs (particularly associated with freshwater) and seagrass diversity, abundance and biomass have been observed elsewhere (Johannes 1980; Kamermans et al. 2002; Mutchler et al. 2007).

As hypothesized, SGD was associated with altered percentage cover of macroalgal and attached macroinvertebrate species in Kinvara bay (Chapter 3) and Olhos de Água (Chapter 4) when compared using multivariate statistical techniques (Table 6-1). The SGD site in Kinvara bay had elevated sessile species richness and greater organism cover compared to the control sites, however this was not statistically significant when individual parameters were compared using univariate statistics (Chapter 3; Table 6-1). Rock pools in receipt of SGD in Olhos de Água, on the other hand, had decreased organism cover (increased bare substrate; Chapter 4) and fewer sessile species than the relative control rock pools (Chapter 4; Table 6-1), though again this was non-significant when compared as discrete parameters using univariate statistics. The disparity in the statistical significance of the results (multivariate versus univariate) highlights the role of multivariate analyses in detecting changes which may go undetected if the individual parameters are compared using univariate statistical methods only (Table 6-1).

Altered structure and composition of the motile macroinvertebrate community was observed at SGD sites in the Ria Formosa lagoon (Chapter 2) and Kinvara bay (Chapter 3), as hypothesised (Table 6-1). SGD was associated with elevated macroinvertebrate biomass and abundance at both sites; however, for both studies only the difference in abundance was statistically significant (Figure 6-1). These results build upon research conducted on diffuse SGD occurring on sand flats, which documented changes in the abundance of motile macroinvertebrate species associated with intertidal SGD in Cape Cod (Dale and Miller 2008; Miller and Ullman 2004), the German Wadden Sea (Zipperle and Reise 2005) and Roscoff Bay in France (Migné et al. 2011a).

In both the Ria Formosa lagoon and Kinvara bay, the difference in the macroinvertebrate assemblage between SGD and control sites was strongly influenced by the occurrence of a small number of rare species which were restricted to either the SGD or the control site. The species that were restricted to the SGD sites only may be used as potential indicator species for SGD in these environments (e.g. *Calyptera chinensis* in the Ria Formosa lagoon (Chapter 2), and *Enchytraeus albidus* and *Elminius modestus* in Kinvara Bay (Chapter 3)). *Gibbula umbilicalis* was also restricted to the SGD site in the Ria Formosa lagoon, and *Littorina littorea* restricted to the SGD site in Kinvara Bay. These latter species are common rocky shore species and thus they cannot be used as indicator species for the presence of SGD, however, their occurrence at the SGD sites and absence from the closely located control sites may indicate that these species show positive selection for SGD sites over similar non-SGD sites where available. This is an area for future research.

6.2. Causative agents of ecological change – stress and disturbance

The environmental factors which control the establishment of primary producers and condition the distribution and success of higher trophic levels are of two types, stress and disturbance. Grime (1979) defined stress as the external constraints which limit production of all or part of the primary producer or distribution of an organism, and disturbance as the mechanisms which limit organism biomass by causing its destruction. A disturbance is a relatively discrete event in time which disrupts the community and population structure of an ecosystem, changing the substrate availability or physical environment (Pickett and White 1985). Generally, disturbances are temporally and spatially heterogeneous, i.e. unpredictable events (Sousa 1979a). SGD can be spatially and temporally variable, however, in the Ria Formosa lagoon (Chapter 2) and Kinvara bay (Chapter 3 and 5), SGD was relatively spatially persistent and the temporal bounds of its occurrence (i.e. frequency, time, duration) somewhat predictable. Thus, SGD may be though of as a form of stress, or a disturbance. In terms of the ecological ramifications of SGD, it is useful and informative to consider

SGD in the context of a disturbance. The idea of SGD as a form of disturbance has been previously suggested (Ouisse et al. 2011). Two main forms of disturbance/stress are proposed to account for the SGD-associated ecological alterations in the current research: (1) salinity; and (2) other non-salinity parameters associated with SGD, in particular in the current research, nitrogen loading. The ecological effects of SGD differed across systems due to differences in the degrees of these disturbances between systems and differential responses of systems to the same disturbances/stressors (Cloern 2001).

6.2.1. Salinity regime and nitrogen loading

SGD was, at least occasionally, of reduced salinity in all three systems (reported herein for Kinvara bay (Chapter 3 and 5) and Olhos de Água (Chapter 4), and reported elsewhere for the Ria Formosa (Leote et al. 2008) (Chapter 2)) (Table 6-1). SGD was nitrogen-enriched relative to the control marine compartments in all three study systems (reported herein for Kinvara bay (Chapter 3 and 5) and Olhos de Água (Chapter 4), and reported elsewhere for the Ria Formosa (Leote et al. 2008) (Chapter 2)) (Table 6-1). SGD reported elsewhere is often of reduced salinity and nitrogen-enriched (e.g. Carruthers et al. 2005; Charette and Buesseler 2004; Hays and Ullman 2007; Leote et al. 2008; Taniguchi et al. 2002; Valiela et al. 1990).

The SGD sites at the three systems studied represent three distinct salinity regimes. The difference in salinity regimes was due largely to differences the physical structure of the systems and flux of freshwater SGD. Both the lagoon (Ria Formosa) and bay (Kinvara) are areas of restricted water exchange, i.e. less than 100% water exchange with each tidal cycle. Olhos de Água, on the other hand, is located on a section of an open coastal system where SGD water is rapidly diluted in the marine water upon its tidal inundation. SGD in the Ria Formosa lagoon was brackish/marine, normally ranging from 20 – 37 psu, though salinities as low as 17 psu have been recorded on occasion (Leote et al. 2008) (Table 6-1). The relatively low flow rate of, where it occurs, brackish SGD (3.6 m^3 day^{-1} per linear meter of coastline (Leote et al. 2008)) and a high hydraulic turnover (Newton and Mudge 2003) support a high and constant salinity in the lagoon (~ 36 psu (Ferreira 2003)). In contrast, a high flow rate (5 – 30 m^3 s^{-1} (Drew 2008)) of fresh groundwater (salinity 0 psu) created a surface freshwater body of salinity 0 at low tide, persisting on occasion even during high tide, at the SGD site in Kinvara Bay. The salinity of rock pools in receipt of SGD in Olhos de Água varied from as low as 4 psu at low tide to ~ 36 psu upon its tidal inundation, with a high frequency of oscillations, i.e. ~ 12 hour tidal cycle (Chapter 3). In short, in the Ria Formosa the SGD sites experienced a largely marine environment and in Kinvara bay a freshwater environment, while the SGD sites at Olhos de Água oscillated between marine and freshwater salinities. Comparison of SGD and control sites within any one study system allows for discernment of the ecological effects of SGD and its associated salinity regime in that particular system. Further, comparison among these three systems allows for the study of the ecological effects of three distinct salinity regimes which occurred due to the presence of SGD.

The influence of salinity over ecological community is well documented. Salinity plays a large role in conditioning the occurrence and proliferation of macroalgae species in estuarine environments (Khfaji and Norton 1979). The dominant species at the SGD sites in the Ria Formosa (*Ulva spp.*) were freshwater tolerant species which tend to occur in areas of nutrient (particularly N) enrichment (Fong et al. 1998). *Fucus ceranoides* was the most abundant species (in terms of cover) at both the SGD and control sites in Kinvara bay, however, cover of this macroalgae was greater (nearly

100%) at the SGD site than the control sites. *F. ceranoides* is widely found in estuaries but absent from fully marine areas (Khfaji and Norton 1979). High salinity presents a stress for this species and thus salinity strongly conditions its occurrence (Khfaji and Norton 1979), while nutrient loading can allow for proliferation and dominance of this species in areas of reduce salinity. *Ulva* spp. were also found in both SGD and relative control pools in the rock pools in Olhos de Água. The occurrence of *Ulva* spp. across the rock pools may be been due to the overflow of nutrient-rich water from the SGD rock pools into the relative control pools. The number of macroalgal species and macroalgal cover were lower in the SGD than the control rock pools. *Ellisolandia elongata* was absent from the SGD rock pools and a negative correlation was found between the cover of *E. elongata* and the fraction of freshwater SGD in the relative control rock pools. *Ellisolandia elongata* is a marine macroalgae which has not been documented in areas of reduced salinity. Thus, it's likely that the salinity regime imposed by the SGD was largely responsible for the observed pattern of *Ellisolandia elongata* distribution in Olhos de Água.

Set suites of macroinvertebrate and fish assemblages occur within certain salinity bands. These bands range from approximately 0-4/5, 2-14, 11-18, 16-27 and > 24 psu (Bulger et al. 1993). The Venice system (1959) identifies comparable bands and also includes a final band at >/=30 psu. As the salinity moves from one band to another, it imposes stress and disturbance on the pre-existing salinity-adapted ecological assemblage (Cañedo-Argüelles and Rieradevall 2010). The effect on the ecological assemblage depends on the frequency of disturbance or change between discrete salinity zones/bands. The salinity may remain in the new band, resulting in the establishment of a new community adapted to the new salinity regime, or it may oscillate between salinity bands with low, intermediate or high frequency.

With reference to the salinity-based zonation developed by Bulger et al. (1993), the ecological assemblage associated with SGD in the Ria Formosa at low tide (salinity as low as 17 psu, but generally 20-37 psu) and lagoon water upon tidal inundation (~36 psu) moves between two salinity zones, the 16-27 and >/=24 psu. This change in salinity bands occurs at low tide, in areas of SGD and only when SGD salinity is <24 psu, that is, not on all occasions when SGD is occurring. This is reflected in the ecology; there was a large degree of overlap of species between the SGD and control areas in this study, as would be expected when there is little difference in the salinity regime between sites. However, there was greatly increased biomass of primary producers at both SGD sites, and, for one beach face section, increased abundance of the most abundant macroinvertebrate across the study at the SGD relative to the control site. It is suggested that this is due to exploitation of SGD delivered nitrogen at the SGD sites. The SGD site in Kinvara Bay experienced a relatively permanent and stable salinity which remained within the 0-4 psu salinity band identified by Bulger et al. (1993). The stress associated with salinity regime at the SGD site in Kinvara bay is expected to be low (due to the low degree of temporal change in salinity). The SGD sites in this case differed in salinity from the control sites; this is again reflected in the ecology, with a smaller proportion of overlapping macroinvertebrate species at the SGD and control sites relative to the Ria Formosa. Similar to the Ria Formosa, the relatively stable salinity conditions enabled organisms at the SGD sites to utilize the SGD-borne N, resulting in greater biomass of primary producers and abundance of macroinvertebrates at the SGD relative to the control site in winter (n.d. in summer). The salinity in the SGD rock pools in Olhos de Água moved from the lowest salinity zone, 0-4 psu , to the highest salinity zone >/= 24 psu (Bulger et al. (1993)). This change occurred during the course of approximately 3 hours (maximal duration of exposure at low tide during low spring tide), at a frequency of approximately 10-12 hours. Such drastic salinity changes would result in a high level of stress/disturbance for the ecological community in the SGD rock pools in Olhos de Água. This is reflect in the ecological assemblage of sessile species where only a small proportion of species found in both the SGD and control rock pools and there were reduced species numbers in the SGD relative to the controls pools.

The highest SGD nitrogen concentrations (at the SGD relative to the control sites/compartments) were found in the rock pools in Olhos de Água and the lowest in the SGD in Kinvara bay (Table 6-1). It might thus have been expected that the greatest primary producer biomass and macroinvertebrate biomass (at SGD sites compared to their relative control sites) would be found in the former system and the lowest of these two parameters in the latter system. This, however, was not the case, indeed, the opposite was found. It is suggested that this anomaly was due to the salinity regime acting as a 'filter' (Cloern 2001) which modulated the ability of the primary producers and subsequent trohpic levels to exploit SGD-delivered N.

Thus, in the Ria Formosa, salinity conditioned to a small degree the species list present, while nitrogen additions and other factors associated with SGD allowed for the proliferation of primary producers and increase abundance of macroinvertebrate species at the SGD sites. In Kinvara bay, the species list was strongly conditioned by the reduced salinity, however, the other factors associated with SGD (particularly N loading) also strongly conditioned the ecological assemblage, allowing for the proliferation of primary producers and increased abundance of macroinvertebrate species at the SGD site. In Olhos de Água, salinity had a very strong influence over the community assemblage and the high frequency of occurrence of the disturbance associated with salinity precluded species from capitalizing on the nitrogen delivered by SGD and subsequent increases in biomass or abundance.

6.2.2. Diversity indices and biotic indices

Decreasing salinity in the marine environment has the effect of reducing the resulting score of diversity indices and biotic indices (Barbone et al. 2012). Barbone et al. (2012) found that the Shanon index and M-AMBI varied significantly with water salinity, with index values decreasing as the salinity moved from euhaline (>30 psu) to polyhaline (18-30 psu), to mesohaline (5-18 psu) and oligohaline (<5 psu). In the Ria Formosa, however, for five of the six comparisons between SGD and control sites, the value of diversity indices were greater at the SGD than the control site. These results are contrary to expectations based on Barbone et al. (2012), but in line with expectations based on the theory that SGD exerted a relatively low level of stress, and in terms of 'disturbance', occurred at an intermediate level of frequency. This maintained a higher species diversity (as indicated by the diversity indices) at the SGD relative to more stable relativel control areas (Sousa 1979b; Sousa 1979a; Connell and Slatyer 1977; Farrell 1991). In this system, the salinity regime allowed SGD nitrogen additions to be exploited by the species present, allowing them to proliferate and have higher biomasses than at the control sites. The diversity indices and raw ecological data indicate that the nitrogen additions delivered by SGD, as well as salinity had a greater influence than salinity in conditioning the ecological community at the SGD sites in the Ria Formosa.

The salinity of water flowing over the control site 2 was 12 psu in Kinvara bay. The biotic indices (metrics) for Kinvara bay indicate a depreciation in ecological status from the control sites to the SGD site. While, this is in line with expectations due to salinity effects (Barbone et al. 2012), for both the Shannon Wiener index and M-AMBI there is no depreciation in index score due to the effect of salinity between 0 and 18 psu (Barbone et al. 2012). Thus, the difference in the Shannon-Weiner and M-AMBI score between the SGD site and control site 2, at least, can be ascribed to the effect of the non-salinity-related disturbance(s) associated with SGD. The Shannon-Wiener index was higher at the control than the SGD site in both seasons. M-AMBI indicated that the SGD site was of 'good status' relative to the control sites which are of 'high status' in both years. M-AMBI has been widely applied in Ireland (Atalah and Crowe 2010; Kennedy et al. 2011), Europe (Muxika et al. 2007) and the US (Borja and Tunberg 2011). It has been successfully applied across the oligohaline to euhaline salinity bands (Borja and Tunberg 2011). The index data, combined with the increased dominance of a small number of species at the SGD site compared to the control are consistent with non-salinity variables associated with SGD presenting a disturbance, which occurs at low frequency (Sousa 1979a). However, given the extreme divergence from marine salinity associated with SGD, it is likely that salinity is the main form of stress/disturbance in this system (where disturbance refers to the complete removal of freshwater SGD followed by presence of SGD). The extreme salinity with very restricted range (0 psu) formed a relatively permanent and stable freshwater environment in the otherwise marine setting. The low frequency of the disturbance presented by salinity allowed sufficient time for the species present to capitalize on the nitrogen additions and resulted in increased macroalgal biomass and macroinvertebrate abundance at the SGD site relative to the control sites.

Species diversity and biotic indices are based on motile macroinvertebrate and fish species, parameters which were not assessed for Olhos de Água. The results of the sessile community survey can be coupled with the known relationships between salinity and ecology (Venice System 1959; Bulger et al. 1993) and the theory of disturbance (e.g. Sousa 1979b) to understand the relationship between SGD and ecology in this system. The SGD entering rock pools in Olhos de Água was the most nitrogen enriched of the three systems studied (Chapter 4; Table 6-1). The fluctuation between a fully marine physicochemical environment upon tidal influx, and an estuarine/freshwater physicochemical environment due to SGD, prohibited colonisation and proliferation of species belonging to any one salinity zone. This resulted in the decreased species diversity and organism cover recorded in the SGD rock pools. The sessile community was also surveyed in Kinvara bay. In Kinvara Bay, in contrast, capitalization by freshwater/estuarine adapted species on resources made available by exclusion of species not adapted to the permanently low and stable salinity resulted in greater sessile species cover at the SGD relative to control sites (Chapter 3; Table 6-1).

6.2.3. Organism biological traits and life histories

The suggested form and frequency of disturbance associated with each system are supported by the selection strategies of the organisms recorded at the systems. MacArthur and Wilson (1967) introduced the terms r- and K-selection to ecology. These groupings divide organisms based on life-history traits, where r-strategist quickly reach maturity (i.e. at an early age), produce a large number of precocial young which require a relatively large reproductive effort overall. K-strategists, on the other hand, reproduce after a longer period of time, and produce a small number of relatively altricial young which require a relatively smaller reproductive effort, overall. Areas which experience disturbance at relatively high or intermediate frequencies generally favour r-selection strategy species (Grimes 1979). Fast growing opportunistic green macroalgae such as *Ulva* spp. and *Cladophora* spp. are r-strategist macroalgae, while brown perennials such as *Fucoids* are K-selection species (Raven and Taylor 2003; Dawes 1998). This is further supported by the life-histories of some of the macroinvertebrates found recorded during the study. *B. reticulatum* found at both the SGD and control sites in the Ria Formosa is a K-selection strategist which belongs to the ecological group 1 of the Biotic Index (BI) discussed early (Borja et al. 2000) – supporting the theory of disturbance occurring at a low to intermediate frequency at SGD sites in this system. Similarly, *Gammarus* spp. and *J. albifrons*, which were found at both the SGD and control sites in Kinvara bay but there *J. albifrons* dominated the SGD site in winter, are also K-strategists (Raberg and Kauksty 2007), supporting the theory of disturbance occurring at a low to intermediate frequency at SGD sites in this system.

Thus, the ecology that developed in association with SGD was conditioned primarily the by salinity regime in Kinvara bay and Olhos de Agua, and by nitrogen loading in the Ria Formosa lagoon. In both the Ria Formosa lagoon (Chapter 2) and Kinvara bay (Chapter 3) nitrogen loading was coupled with increased primary producer biomass at the SGD sites. This may indicate early signs of the onset of eutrophication, particularly so when associated with altered macroinvertebrate species composition and increased biomass/abundance of a restricted species list (Warwick 1986), as was observed in the macroinvertebrate assemblage at SGD sites in Kinvara bay (Chapter 3). This research may provide support for eutrophication-concern based management of SGD in these two systems.

The difference in the macroinvertebrate assemblage between SGD and control sites in both Ria Formosa lagoon and Kinvara bay was strongly influenced by the occurrence of a small number of rare species which were restricted to either the SGD or the control site. The species that were restricted to the SGD sites only may be used as potential indicator species for SGD in these environments (e.g. *Calyptera chinensis* in the Ria Formosa lagoon (Chapter 2), and *Enchytraeus albidus* in Kinvara Bay (Chapter 3)). *Gibbula umbilicalis* was also restricted to the SGD site only in the Ria Formosa lagoon, and *Littorina littorea* restricted to the SGD site in Kinvara Bay. These latter species are common rocky shore species. While these species cannot be used as indicator species for the presence of SGD, their occurrence at the SGD sites and absence from the closely located control sites may indicate that these species show positive selection for SGD sites over similar non-SGD sites where available. This is an area for future research.

The presence of SGD resulted in spatial heterogeneity that facilitated the occurrence of two discrete habitats, which supported discrete food webs at the small/medium spatial scale in both the Ria Formosa lagoon (~100 m), and Kinvara bay (~100 and ~300 m). The presence of two discrete food webs with some non-overlapping species resulted in increased biodiversity at a larger spatial scale. This elevated biodiversity may bring with it the concomitant benefits generally ascribed to increased biodiversity, such as increased resilience and stability (Cardinale et al. 2012). SGD can be a major modulating agent for the structure, composition and development of benthic intertidal food webs in intertidal sand flat and rocky systems. Though SGD was consistently associated with ecological alterations, the form of the alterations differed across systems, conditioned by the attributes of disturbance(s) and the system responses to these disturbances. The effects of SGD on ecology are conditioned by a number of physical parameters (SGD flow rate and degree of restriction of water exchange in receiving system, if any) and physicochemical properties of the SGD, particularly salinity and nitrogen concentrations. Where it occurs, omitting the potential influence of SGD over ecology (particularly the freshwater compartment) may lead to errors when interpreting the results of ecological surveys, particularly in karst systems.

6.2. Stable isotope analysis (SIA) in SGD research

It was hypothesized that the nitrogen isotopic signature of SGD food web components would differ from that of their control food web counterparts, and similarly, that the carbon isotopic signature of SGD food web components would differ from that of their control food web counterparts. Algae sampled from the SGD area on beach face section B in the Ria Formosa had lower δ^{15}N values and higher δ^{13}C values relative to algae sampled from the control area. *Bittium reticulatum* tissue sampled at the SGD areas in the Ria Formosa was depleted in ^{15}N and enriched in ^{13}C relative to the control sites; these differences were less pronounced than for algae. Considering all isotopic comparisons at the Ria Formosa, only for δ^{15}N of *B. reticulatum* sampled from beach face section B was the difference statistically significant. In both seasons, macroalgae at the SGD site in Kinvara bay were statistically significantly ^{13}C depleted relative to macroalgae at the control site. Macroalgal ^{15}N in Kinvara bay differed statistically between the SGD and control site in summer and macroinvertebrate ^{13}C and ^{15}N values differed statistically between the SGD and control site in winter. Thus the isotopic patterns and results were clearer for Kinvara bay than the Ria Formosa lagoon, and the original hypotheses could be only partially rejected. Not withstanding this, stable isotope analysis of oxygen, nitrogen and carbon was used to distinguish two food webs in the Ria Formosa lagoon (Chapter 2) and Kinvara bay (Chapter 5), where one food web primarily utilised SGD-borne N and C, and the other primarily utilised other non SGD-borne C and N. SIA indicated SGD-associated N loading in both systems, and C loading in Kinvara bay. Macroalgal and macroinvertebrate $\delta^{13}C$ values indicated that SGD enhanced the rate of carbon turnover and primary producer respiration at SGD areas in the Ria Formosa lagoon (Chapter 2). The ^{15}N data in the Ria Formosa lagoon was used to infer (a) some influence by a groundwater source and (b) a synthetic fertilizer N source to the SGD food web (Chapter 2). In Kinvara bay, $\delta^{15}N$ values were used to infer SGD N loading in Kinvara bay with some N contribution to the SGD food webs from a septic tank effluent/wastewater N source (Chapter 5). The $\delta^{13}C$ values of macroalgae sampled at the SGD site were unusually low, particularly in winter. This was used to indicate that SGD was a source of carbon loading in Kinvara bay. Due to greater divergences in $\delta^{13}C$ values between the SGD and relative control sites, $\delta^{13}C$ values provided a more reliable indicator than $\delta^{15}N$ values in identifying and tracing pathways of N and C trophic transfer through SGD and relative control food webs in Kinvara bay. SIA linked the nitrogen in SGD to the nitrogen in the associated coastal food web, implicating SGD borne nutrients of terrestrial, and possibly of anthropogenic origin, in the functioning of intertidal ecosystems. This may provide a method of identifying incipient eutrophication in SGD systems, enabling early intervention and remediation.

Stable isotope ratios of carbon and nitrogen are frequently used in coastal habitats to discriminate which primary producers support the food web. In the current research, however, the primary producers supporting the food webs under comparison were of the same species (*Ulva* spp. in the Ria Formosa lagoon (Chapter 2) and *F. ceranoides* and *Ulva* spp. in Kinvara bay (Chapter 5)). Thus, here, instead, SIA provided a means of discriminating from which of two water sources occurring in the same environment (SGD or marine) the food web sourced its nutrients (N primarily, but also C). Further, SIA allowed discernment of the terrestrial source of the SGD-borne N.

This research represents the second time that SIA of organism tissue has been used to study food web dynamics associated with intertidal SGD. Both Ouisse et al. (2011) and the current research found that $\delta^{13}C$ and $\delta^{15}N$ values of food web components differed between SGD and control areas, however, the results are somewhat ambiguous and not applicable across all sites. SIA may provide a useful tool for identifying SGD-dependant food webs, and investigating the role of SGD in nutrient trophic transfer. SIA may be used to trace SGD-borne N into and through SGD-reliant food webs, and carbon isotopic signatures may be used to inform upon alterations to food web metabolism and nutrient cycling associated with SGD.

Analysis of $\delta^{15}N$ and particularly $\delta^{13}C$ values of higher trophic level organisms may be extended to determine the relative importance of SGD in compromising or sustaining ecosystem functioning and thus, ecosystem goods and services. For this, $\delta^{13}C$ values may provide a more useful tracer than $\delta^{15}N$ values as they displayed greater divergence in signature between SGD and control food web components, for both the Ria Formosa lagoon and Kinvara bay (Chapter 2 and 5 respectively). Also, the trophic enrichment of ^{13}C is relatively well defined and negligible (~ 0 to +1 ‰ (DeNiro and Epstein 1978; Peterson and Fry 1987)), allowing for relatively easy interpretation of results compared to ^{15}N for which there is a greater range in trophic enrichment values, ~ +2.5 ‰ to +3.5 ‰ (Zanden and Rasmussen 2001; Vanderklift and Ponsard 2003; DeNiro and Epstein 1981). Organism $\delta^{13}C$ values may provide a means of tracing and studying SGD where the $\delta^{15}N$ value of the SGD and marine water are similar, irrespective of the presence of nitrogenous pollution. Stable carbon isotopes are particularly useful in the case of a freshwater SGD component as the isotopic signature of freshwater-borne DIC is distinct (lower than) that of marine DIC.

As traditional estuary and lagoon transitional coastal waters are characterised by strong gradients, high variability in abiotic factors and high biological productivity (Basset et al. 2013), so too are SGD associated transitional waters, mainly where SGD is freshwater in form and profuse in delivery. The high degree of environmental variability stemming from these features poses problems for monitoring their ecological status and thus management and conservation (Basset et al. 2013). SIA may be used to elucidate numerous aspects of SGD associated ecology, as well as other processes such as carbon acquisition by macroalgae and carbon cycling in coastal environments. Stable isotope analysis of organisms is a nearly unused method in SGD research, however, as the current research illustrates, this technique holds potential to further our understanding of SGD and broader coastal ecosystem functioning.

6.3. Future research

Karst-channeled SGD is saturated in dissolved inorganic carbon due to carbonate dissolution by carbonic acid, nitric acid and sulfuric acid in rainwater. While the open ocean is a net sink for CO_2 (Chen and Borges 2009), most estuaries and near-shore coastal areas (mainly mangroves and salt marshes) are a net source of inorganic carbon (Chen and Borges 2009; Fagan and Mackenzie 2007). SGD is a demonstrated source of coastal CO_2, with karst-channelled SGD particularly relevant in this context due to its high DIC load. Dorsett et al. (2011) estimated that SGD accounts for up to 28 % of water column DIC in the Indian River Lagoon along the Floridian coast, and from this, estimated that 7 – 11 % of global coastal water DIC may be derived from SGD associated with karst and other carbonate systems. The altered coastal DIC concentrations and speciation due to freshwater SGD, and particularly karst-channeled

SGD might, however, potentially deleteriously affect carbonate exoskeleton-bearing species such as marine calcifiers, or coralline algae. The positive correlation between salinity and cover of the coralline algae *E. elongata* found in the Portuguese rock pools may be an example of this and presents an area for future research (Chapter 4). To date, no research exists describing the role of SGD in conditioning the occurrence or distribution of *E. elongata.*

The greatly ^{13}C depleted algae sampled at the SGD site in winter in Kinvara Bay may have implications for the interpretation of algal and herbivore δ^{13}C values in coastal ecological research in areas in receipt of SGD, particularly karst-channeled SGD. It is suggested that algae sampled from the SGD site in Kinvara bay in winter were depleted in ^{13}C due to a glut of dissolved CO_2 delivered by SGD. Such algal ^{13}C depletions have until now been observed in only a very restricted number of genera, mostly within the Rhodophyta (Maberly et al. 1992; Raven et al. 1995; Raven et al. 2002). Generally, in intertidal and coastal research, ^{13}C depletion in faunal tissues is attributed to a terrestrial organic carbon source. The results herein may however indicate that very low δ^{13}C values possibly also derive from carbon fixed in the marine environment associated with [CO_2] inputs, perhaps delivered by SGD. This has implications for (1) studies of coastal food web C use and transfer in systems in receipt of freshwater SGD, and karst-channeled SGD in particular, (2) our understanding of coastal food web resource use, and (3) the cycling of carbon between terrestrial and marine environments. Further research should be conducted using SIA to elucidate the role of SGD in the carbon cycle and the effect of SGD on macroalgal isotopic signatures, particularly in karst and carbonate systems. Where SGD is associated with C loading, algal δ^{13}C values may be used as a proxy to indicate SGD DIC concentration and C loading, similar to Lehmann et al. (2004). If a clear relationship can be established between SGD DIC concentration and the $\delta^{13}C$ of macroalgae using SIA, algal $\delta^{13}C$ may (a) provide an indicator of the coastal and oceanic carbon loading associated with SGD, (b) inform on the geographical and vertical extent of the impact of this C loading source, and (c) act as a potential discriminatory technique allowing distinction between SGD and atmospheric-derived CO_2 in coastal and oceanic settings.

Assisted by the relatively conservative nature of nitrate transport through well-oxygenated karst aquifers (Slomp and Van Cappellen 2004), karst-channeled SGD delivered high nitrogen fluxes to the SGD rock pools in Olhos de Água (Chapter 4), and Kinvara bay SGD sites (Chapter 2). The SGD in Olhos de Água was nitrogen enriched by both marine and groundwater standards. In Kinvara bay, SGD was N-enriched only relative to marine thresholds. There is, however, no set threshold as to what constitutes 'N-contaminated', or 'N-enriched' *SGD*. This highlights the need for definition of SGD nutrient (particularly N) maximum allowable concentration (MAC) thresholds in light of their ecological impact in the receiving marine environment, and perhaps also revision of N thresholds for the groundwater bodies which fuel SGD.

Paradigms should be constantly reviewed in the light of new information and understanding, and in particular, current paradigms need to be tested, rejected or reaffirmed in estuarine ecology and management (Elliott and Whitfield 2011). This consideration may sometimes challenge our view of a topic and even give new directions for future research (Elliott and Whitfield 2011). In light of the relatively recent recognition of SGD as a marine ecological process and emerging knowledge regarding the estuarine systems which it can underpin, individual cases of SGD should be reviewed in light of current paradigms. Ecological paradigms can be developed by their consideration in light of SGD, increasing our understanding of these principles and

SGD as an ecological process. One such paradigm is, for example, the role SDG plays in modulating the typical vertical zonation of rocky intertidal food web structure. Vertical zonation was not observed in any system in the current research. Substantial research has been invested in determining the causes of distribution patterns, particularly, zonation of algae and sessile animals on rocky shores. In the 1970s, a paradigm developed that upper limits were set by physical factors and lower limits by biological interactions (Connell 1972). This theory was tested along a Portuguese limestone coast where it was found that the occurrence of red algal turf was determined by grazing pressure (Boaventura et al. 2002). In the absence of limpet grazers, red algal turfs proliferated, while areas that supported grazers were devoid of algae (Boaventura et al. 2002). Though usually not as pronounced in rock pools as on emergent substrata (Metaxas and Scheibling 1993), vertical zonation was absent at all sites in the current study. The ability of SGD to interrupt and alter this pattern of zonation has not been assessed. SGD might, for example, create favorable or unfavorable conditions for grazers, thus altering their distribution with a concomitant effect on the macroalgal assemblage. Alternatively, SGD might create an unfavorable environment for the algae (as was seen in the case of *Ellisolandia elongata* in the rock pools at Olhos de Água (Chapter 4)), influencing the pattern and distribution of both algae and the species which depend on it for food and habitat. The influence of intertidal SGD, and freshwater SGD in particular, in conditioning patterns of zonation has yet to be examined.

The catchment of Olhos de Água receives a relatively high loading of nitrogenous pollution, derived mostly from farming activities and tourism (Stigter et al. 2011). The catchment of Kinvara bay on the other hand is subject to relatively low levels of nitrogen pollution due to low population density and relatively low levels of tourism and agriculture (CSO 2011). SGD, however, resulted in nitrogen loading in both systems. Thus, SGD can be a source of coastal nitrogen loading even when associated with a catchment subject to relatively low intensity of anthropogenic usage and nitrogenous pollution. Particularly, terrestrial anthropogenic nitrogen application/pollution is more closely coupled with coastal SGD nitrogen loading for karst-channeled SGD than for other hydrogeological settings. Given the ubiquity of karst and carbonate coastal systems (accounting for 25% of the worlds coastline (Ford and Williams 2007)) and karst-channeled SGD, particular attention should be given to karst-channeled SGD in future research on the ecological alterations associated with SGD, even in catchments with low intensity of anthropogenic pressure and nitrogen application.

7. References

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8. Appendices

Appendix A - Sampling sites

Appendix A-1 The Ria Formosa (Chapter 2)

Figure 8-1.1 Beach face A – SGD area

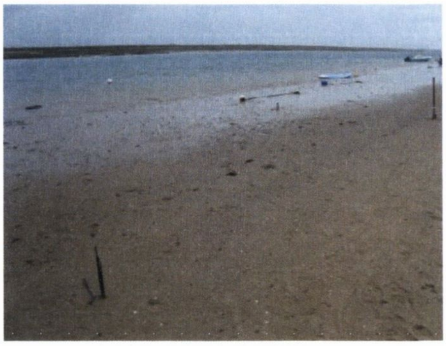

Figure 8-1.2 Beach face A – control area

Figure 8-1.3 Beach face A – SGD area

Figure 8-1.4 Beach face A – control area

Figure 8-1.5 Beach face B – SGD area

Figure 8-1.6 Beach face B – control area

Appendix A-2 Kinvara Bay (Chapter 3 and 5)

Figure 8-2.1SGD site 1

Figure 8-2.2 SGD site 1.Fucus ceranoides line extends from the water line to the grass, with no intermediate zones

Figure 8-2.3 SGD site 1

Figure 8-2.4 Quadrat survery at SGD site 1

Figure 8-2.5 Quadrat survery at SGD site 1

Figure 8-2.6 Control site 2 (summer 2011)

Figure 8-2.7 Quadrat survey at control site 2 (summer 2011)

Figure 8-2.8 Control site 3 (winter 2012)

Figure 8-2.9 Control site 3 (winter 2012)

Appendix A-3 Olhos de Agua karst plateau and rockpools (Chapter 4)

Figure 8-3.1 View from land, oceanward

Figure 8-3.2 Rockpool 9

Figure 8-3.3 Largest rockpool on plateau

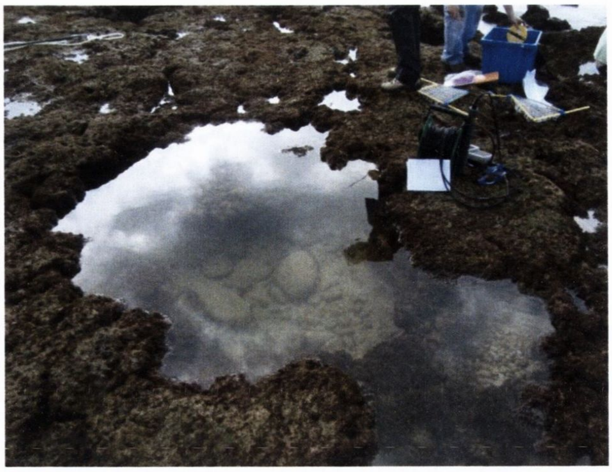

Figure 8-3.4 Deepest rockpool measured

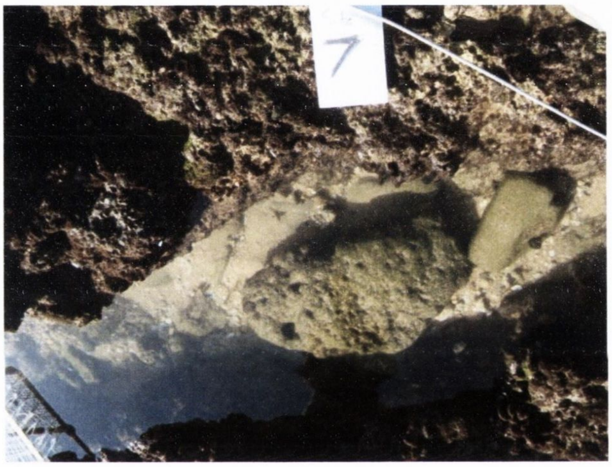

Figure 8-3.5 Rockpool 7

Appendix B - Organism Illustrations

Appendix B-1 The Ria Formosa (Chapter 2)

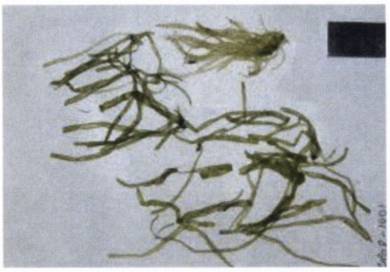

Figure 8-4.1 ***Ulva intestinalis***

Figure 8-4.3 ***Ulva rigida***

Figure 8-4.5 ***Colpomenia peregrina***

Figure 8-4.7 ***Bostrychia scorpioides***

Figure 8-4.2 ***Bittium reticulatum***

Figure 8-4.4 ***Tapes decussates***

Figure 8-4.6 ***Hydrobia ulvae***

Figure 8-4.8 ***Cerastoderma edule***

Figure 8-4.9 *Phascolion strombi*

Figure 8-4.10 *Melita palmata*

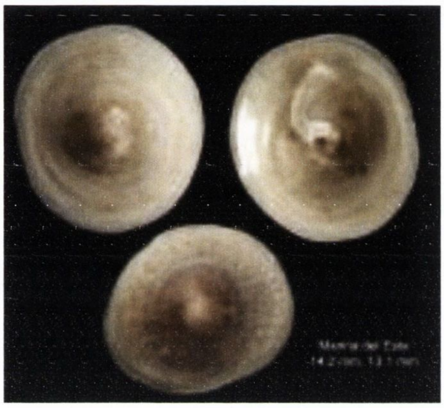

Figure 8-4.11 *Calyptraea chinensis*

Figure 8-4.12 *Gibbula umbilicalis*

Figure 8-4.13 *Anguilla anguilla*

Figure 8-4.14 *Cyathura carinata*

Appendix B-2 Kinvara Bay (Chapter 3)

Figure 8-5.1 ***Polysiphonia*** **spp. (the red epiphytic algae)**

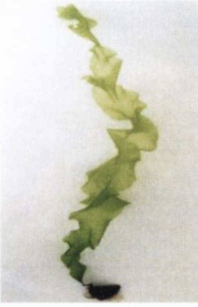

Figure 8-5.2 ***Ulva linza***

Figure 8-5.3 ***Cladophora rupestris***

Figure 8-5.4 ***Elminius modestus***

Figure 8-5.5 ***Ascophullum nodosum***

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Figure 8-5.6 ***Jaera albifrons***

Figure 8-5.7 ***Ulva intestinalis***

Figure 8-5.8 ***Chaetogammarus marinus***

Figure 8-5.9 *Corophium volutator*

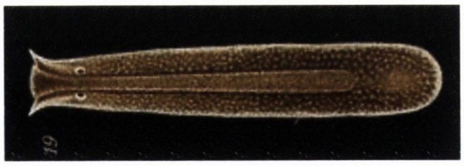

Figure 8-5.10 *Procerodes littoralis*

Figure 8-5.11 *Enchytraeus albidus*

Figure 8-5.12 *Littorina littorea*

Figure 8-5.13 *Carcinus maenus*

Figure 8-5.14 *Ostracoda sp.*

Figure 8-5.16 *Fucus ceranoides*

Figure 8-5.15 *Chaetomorpha linum*

Appendix B-3 Olhos de Agua (Chapter 4)

Figure 8-6.1 ***Mytilus galloprovincialis***

Figure 8-6.2 ***Gelidium latifolilum***

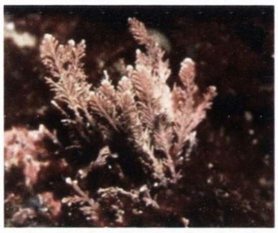

Figure 8-6.3 ***Ellisolandia elongata***

Figure 8-6.4 ***Gigartina acicularis***

Figure 8-6.5 ***Chordaria flagelliformis***

Figure 8-6.6 ***Cystoclonium perpureum***

Figure 8-6.7 ***Dictyota dichtonia***

Figure 8-6.8 ***Plocamium cartilagineum***

Appendix C - Raw Data

Appendix C-1 The Ria Formosa (Chapter 2) raw data

Table 8-1.1 Algal biomass at impacted site S1 and S4 and control sites S2 and S3, where sample event is identified by 'E' number

	S1E1	S1E2	S1E3	S1E4	S1E5	S2E1	S2E2	S2E3	S2E4	S2E5
Ulva rigida	0.08	2.343	0	1.355	1.0178	0	0	0	0	0
Ulva intestinalis	1.56	1.116	0	4.182	2.0923	0	0	0	0	0
Colpomenia peregrina	0	0	0	0	0	0	0	0	0	0
Bostrychia scorpioides	0	0	0	0	0	0	0	0	0	0

	S3E1	S3E2	S3E3	S3E4	S3E5	S4E1	S4E2	S4E3	S4E4	S4E5
Ulva rigida	0	0.0313	0	0.0436	0	0	0.0352	0.1611	0.0596	0.3178
Ulva intestinalis	0.14	0.0663	0.0527	0.0979	0.0338	0.0442	0.2753	0.0491	0.3259	0.8789
Colpomenia peregrina	0	0	0	0	0	0.0144	0	0	0	0.1972
Bostrychia scorpioides	0	0	0	0	0	0	0	0.3127	0.037	0.0592

Table 8-1.2 Macroinvertebrate abundance at impacted site S1 and control site S2, where sample event is identified by 'E' number

	S1E 1	S1E 2	S1E 3	S1E 4	S1E 5	S2E 1	S2E 2	S2E 3	S2E 4	S2E 5
Acanthochitona crinitus	0	0	0	0	0	0	0	0	0	0
Amphipholis squamata	0	0	0	4	1	0	0	0	0	0
Anguilla anguilla	0	0	0	0	0	0	0	0	0	0
Bittium reticulatum	4	6	10	20	1	0	11	142	66	0
Calyptraea chinensis	0	1	0	0	0	0	0	0	0	0
Carcinus maenas	0	1	0	0	0	0	0	0	0	0
Cerastoderma edule	2	9	3	3	0	2	0	2	1	4
Clibanarius erythropus	0	0	0	1	0	0	0	0	0	0
Cumacean	0	0	2	0	0	0	0	1	0	0
Cyathura carinata	0	1	0	0	0	0	0	0	0	0
Cyclope spp.	0	0	0	0	0	0	0	0	0	0
Gibbula umbilicalis	0	4	0	0	0	0	0	0	0	0
Gibbula varia	0	0	0	1	0	0	0	0	0	0
Hydrobia ulvae	0	0	0	1	2	1	1	0	1	0
Anguilla anguilla	0	0	0	2	0	0	0	0	0	0
Melita palmata	0	6	0	3	1	0	0	0	0	0
Mesalia brevialis	1	0	0	0	0	0	0	0	0	0
Nassarius reticulatus	2	1	0	6	0	0	0	0	0	0
Ophiura ophiura	0	0	0	0	0	0	0	1	0	0
Owenia fusiformis	0	0	0	0	0	0	0	1	0	0
Paguristes spp.	0	0	0	0	0	0	0	1	1	0
Phascolion strombi	0	0	0	0	1	0	0	0	1	0
Sphaeromatid	0	0	0	0	0	0	0	0	1	0
Spisula subtruncata	0	0	0	0	0	0	0	0	0	0
Tapes decussatus	0	0	0	1	0	0	0	1	0	1
Turritella communis	0	0	0	0	0	0	0	1	0	0
Venus verrucosa	0	0	0	0	0	0	0	1	0	0

Table 8-1.3 Macroinvertebrate abundance at impacted site S1 and control site S2, where sample event is identified by 'E' number

	S3E1	S3E2	S3E3	S3E4	S3E5	S4E1	S4E2	S4E3	S4E4	S4E5
Acanthochitona crinitus	0	0	0	0	0	0	1	0	0	1
Amphipholis squamata	0	0	0	0	0	0	0	0	0	0
Anguilla anguilla	0	0	0	0	0	0	0	0	0	0
Bittium reticulatum	11	8	6	4	5	31	93	38	394	260
Calyptraea chinensis	0	0	0	0	0	1	0	0	1	1
Carcinus maenas	0	0	0	0	0	0	0	0	0	0
Cerastoderma edule	0	0	1	0	1	0	0	0	0	0
Clibanarius erythropus	0	0	0	0	0	0	0	0	0	0
Cumacean	0	0	0	0	0	1	1	0	1	0
Cyathura carinata	0	0	0	0	0	0	0	1	0	0
Cyclope spp.	0	0	1	4	0	1	0	0	1	1
Gibbula umbilicalis	0	0	0	0	0	0	1	0	2	1
Gibbula varia	0	0	0	0	0	0	0	0	0	0
Hydrobia ulvae	3	5	18	1	13	5	5	12	5	5
Anguilla anguilla	0	0	0	0	0	0	0	1	0	0
Melita palmata	0	0	0	0	0	0	0	1	0	0
Mesalia brevialis	0	0	0	0	0	0	0	0	0	0
Nassarius reticulatus	0	0	0	0	0	0	0	0	0	0
Ophiura ophiura	0	0	1	0	0	0	0	0	0	0
Owenia fusiformis	0	0	0	0	0	0	0	0	0	0
Paguristes spp.	0	0	0	0	0	0	1	3	2	0
Phascolion strombi	0	0	0	0	0	2	0	4	14	3
Sphaeromatid	0	0	0	0	0	0	3	0	0	1
Spisula subtruncata	0	0	0	0	0	0	0	0	1	0
Tapes decussatus	0	0	0	0	0	1	1	0	1	0
Turritella communis	0	0	0	0	0	0	0	0	0	0
Venus verrucosa	0	0	0	0	0	0	0	0	0	0

Table 8-1.4 Macroinvertebrate biomass at impacted site S1 and control site S2, where sample event is identified by 'E' number

	S1E1	S1E2	S1E3	S1E4	S1B5	S2E1	S2E2	S2E3	S2E4	S2E5
Bittium reticulatum	0.063	0.118	0.135	0.379	0.017	0.000	0.079	1.173	0.872	0.000
Cerastoderma edule	28.00	202.950	55.350	53.750	0.000	5.151	0.000	6.606	2.270	6.813
Melita palmata	0	0.01357	0	0.0069	0.0061	0	0	0	0	0
Nsarrarius reticulatus	3.090	0.780	0.000	8.885	0	0	0	0	0	0
Amphipolus squamata	0	0	0	0.0076	0.0019	0	0	0	0	0
Cumacean	0	0	0.003	0	0	0	0	0.0015	0	0
Juvenile eel	0	0	0	0.6665	0	0	0	0	0	0
Hydrobia ulvae	0	0	0	0.0121	0.0242	0.0096	0.0053	0	0.0067	0
Phascoion strmbi	0	0	0	0	0.0001	0	0	0	0.0001	0
Calyptera chinensis	0	0.42	0	0	0	0	0	0	0	0
Cyanathura carcina	0	0.0001	0	0	0	0	0	0	0	0
Mesalia brevilis	0.0001	0	0	0	0	0	0	0	0	0
Gibbula varia	0	0	0	0.1665	0	0	0	0	0	0
Gibbula umbilicalis	0	1.43	0	0	0	0	0	0	0	0
Tapes deccusates	0	0	0	3.1232	0	0	0	0.2092	0	0.7168
Clibanarius erytro	0	0	0	0.008	0	0	0	0	0	0
Neries diversicolor	0	0	0	0	0	0	0	0	0	0
Carcinus maenus	0	0.2506	0	0	0	0	0	0	0	0
Paguriste	0	0	0	0	0	0	0	0.002	0.002	0
Sphaeroma spp	0	0	0	0	0	0	0	0	0.0087	0
Owenia fusiforma	0	0	0	0	0	0	0	0.0001	0	0
Venus verrocos	0	0	0	0	0	0	0	0.0001	0	0
Turitella communis	0	0	0	0	0	0	0	0.6821	0	0
Cyclope sp	0	0	0	0	0	0	0	0	0	0

Ophiura ophiura	0	0	0	0	0	0	0	0	0	0
Acanthonchitonia	0	0	0	0	0	0	0	0	0	0
Spisula subtruncata	0	0	0	0	0	0	0	0	0	0

Table 8-1.5 Macroinvertebrate biomass at impacted site S1 and control site S2, where sample event is identified by 'E' number

	S3E1	S3E2	S3E3	S3E4	S3E5	S4E1	S4E2	S4E3	S4E4	S4E5
Bittium reticulatum	0.098	0.156	0.049	0.025	0.040	0.522	1.396	0.368	4.704	9.0168
Cerastoderma edule	0	0	2.2914	0	3.112	0	0	0	0	0
Melita palmata	0	0	0	0	0	0	0	0.0025	0	0
Nsarrarius reticulatus	0	0	0	0	0	0	0	0	0	0
Amphipolus squamata	0	0	0	0	0	0	0	0	0	0
Cumacean	0	0	0	0	0	0.0015	0.0015	0	0.0015	0
Juvenile eel	0	0	0	0	0	0	0	0.0333	0	0
Hydrobia ulvae	0.0286	0.0434	0.1683	0.0105	0.1204	0.036	0.04	0.0792	0.0367	0.0376
Phascoion strmbi	0	0	0	0	0	0.0002	0	0.0004	0.0014	0.0003
Calyptera chinensis	0	0	0	0	0	0.151	0	0	0.1665	0.044
Cyanathura carcina	0	0	0	0	0	0	0	0.0001	0	0
Mesalia brevilis	0	0	0	0	0	0	0	0	0	0
Gibbula varia	0	0	0	0	0	0	0	0	0	0
Gibbula umbilicalis	0	0	0	0	0	0	0.0451	0	0.318	0.121033
Tapes deccusates	0	0	0	0	0	0.0897	0.4532	0	0	0.292
Clibanarius erytro	0	0	0	0	0	0	0	0	0	0
Neries diversicolor	0	0	0	0	0	0	0	0	0	0
Carcinus maenus	0	0	0	0	0	0	0	0	0	0
Paguriste	0	0	0	0	0	0	0.002	0.006	0.004	0

Sphaeroma spp	0	0	0	0	0	0.0174	0	0	0	0.0058
Owenia fusiforma	0	0	0	0	0	0	0	0	0	0
Venus verrocos	0	0	0	0	0	0	0	0	0	0
Turitella communis	0	0	0	0	0	0	0	0	0	0
Cyclope sp	0	0	0.1683	0.2508	0	0.351	0	0	0.2041	0.2929
Ophiura ophiura	0	0	0.5649	0	0	0	0	0	0	0
Acanthonchitonia	0	0	0	0	0	0	0.002	0	0	0.0314
Spisula subtruncata	0	0	0	0	0	0	0	0	0.2281	0

Table 8-1.6 Algal isotope and elemental results for impacted site S1

	C%	δ^{13}C	N%	δ^{15}N	C/N
S1	16.05	-14.03	1.76	6.61	10.61
S1	13.87	-15.66	1.56	6.44	10.38
S1	13.50	-15.72	1.60	7.26	9.81
S1	21.20	-16.04	2.83	6.56	8.74
S1	13.34	-15.78	1.61	6.64	9.63
S1	16.02	-14.26	1.78	6.94	1049
S1	10.39	-15.90	1.29	7.14	9.36
S1	15.28	-14.64	1.67	7.22	10.64
S1	11.51	-16.24	1.38	6.13	9.74
S1	15.73	-14.44	1.85	6.55	9.92

Table 8-1.7 Algal isotope and elemental results for impacted site S4 and control site S3

	C%	δ^{13}C	N%	δ^{15}N	C/N
S3	11.63	-17.02	1.17	7.37	11.55
S3	11.75	-17.25	1.25	7.67	10.94
S3	14.06	-17.38	1.55	7.59	10.57
S4	18.01	-15.12	1.86	7.13	11.29
S4	10.86	-15.94	1.34	6.70	9.45
S4	15.53	-15.82	1.76	7.01	10.29
S4	10.09	-15.57	1.14	6.44	10.31
S4	10.58	-14.79	1.17	7.04	10.52
S4	12.85	-16.72	1.27	7.40	11.76
S4	14.74	-15.57	1.73	6.97	9.96
S4	18.74	-17.24	2.41	7.69	9.08
S4	24.80	-13.45	2.03	7.35	14.24
S4	15.56	-16.01	1.81	7.02	10.04
S4	13.69	-15.80	1.60	7.24	9.98
S4	18.65	-15.09	2.11	7.16	10.29
S4	19.85	-14.90	2.26	6.83	10.25

Table 8-1.8 *Bittium reticulatum* isotopic and elemental results at impacted sites S1 and S4, and control site S2

	C%	δ^{13}C	N%	δ^{15}N	C/N
S1	2.66	-14.06	0.64	8.46	4.83
S1	2.24	-14.31	0.54	7.82	4.86
S1	2.51	-13.86	0.61	9.61	4.76
S1	2.56	-14.14	0.61	8.42	4.87
S1	2.63	-14.39	0.66	8.19	4.61
S1	2.19	-14.26	0.55	8.11	4.67
S2	2.71	-13.94	0.67	7.43	4.71
S2	2.26	-13.92	0.5	8.32	4.8
S2	2.93	-14.24	0.7	7.48	4.7
S2	2.75	-14.02	0.7	7.63	4.8
S2	2.65	-14.13	0.6	8.08	4.9
S3	2.34	-13.68	0.59	8.06	4.59
S3	2.01	-14.12	0.52	8.11	4.51
S3	2.92	-13.82	0.66	8.64	5.2
S3	1.84	-13.88	0.48	7.44	4.5
S3	2.1	-14.11	0.54	8.59	4.5
S4	2.4	-11.73	0.5	7.04	5.7
S4	2.6	-14.06	0.6	6.3	5.5
S4	3.7	-14.87	0.5	5.25	9.1
S4	1.8	-13.64	0.3	7.76	6.1
S4	2.4	-8.599	0.6	7.8	4.6

Appendix C-2 Kinvara Bay raw data (Chapter 3)

Table 8-2.1 N as nitrate and nitrite ($NO_3^- + NO_2^-$)

Summer		
	ug/L	µM
GW	917	65.50
	963	68.79
	921	65.79
SGD (S1)a	884	63.14
	827	59.07
	818	58.43
	816	58.29
SGD/marine	797	56.93
	903	64.50
	890	63.57
	850	60.71
	860	61.43
SGD (S1)b	976	69.71
	847	60.50
	829	59.21
SGD/Marine	373	26.64
	424	30.29
	434	31.00
	400	28.57
	408	29.14
Control (S2)	216	15.43
	218	15.57
	222	15.86
	219	15.64

Winter		
	ug/L	µM
SGD (S1)ht	1590	113.57
	1400	100.00
	1290	92.14
SGD (S1)c	1150	82.14
	1120	80.00
	1130	80.71
SGD (S1)d	1250	89.29
	1250	89.29
	1240	88.57
Galway bay	145	10.36
	141	10.07
GW	840	60.00
	841	60.07
	846	60.43

Table 8-2.2 Water physicochemical parameters

		Salinity (psu)	pH	Temperature (°C)
Summer	GW	0	7.21	16.4
	SGD (S1)	0.1	6.94	11.8
		0.5	7.28	12.3
		0	6.98	11.8
		0	7.04	11.6
	SGD/marine	0	7.07	11.2
	Control (S2)	13.9	8.13	15.4
		13	8.17	15.4
		10.8	7.97	14.7
		10.8	7.94	14.3
Winter	GW	0	7.4	11.6
	SGD(S1)	0	7.18	11.3
		0	7.41	11.4
	Galway Bay	33	8.2	10

Table 8-2.3 Macroalgal and sessile macroinvertebrate percentage cover

	Summer									
	SGD (S1)					Control (S2)				
	Q1	Q2	Q3	Q4	Q1	Q1	Q2	Q3	Q4	Q5
Substrate	7.2	0.0	2.5	3.7	11.2	18.5	8.8	16.0	8.0	16.2
Fucus ceranoides	85.6	83.3	79.3	69.4	60.0	36.4	36.5	61.1	52.3	49.3
Unid. spp. 2	3.0	0.8	0.0	0.8	8.8	0.0	0.0	0.0	0.0	0.0
Unid. spp. 3	0.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Ulva linza/ U. intestinalis	3.6	5.0	1.7	3.0	3.2	39.9	54.7	22.9	26.5	14.8
Unid. spp. 1	0.0	0.0	0.0	6.0	8.0	0.0	0.0	0.0	0.0	0.0
Unid. spp. 4	0.0	0.0	0.0	0.0	3.2	0.0	0.0	0.0	0.0	0.0
Elminius modestus	0.0	10.8	16.5	17.2	5.6	0.0	0.0	0.0	0.0	0.0
Mastocarpus stellatus	0.0	0.0	0.0	0.0	0.0	0.0	5.2	0.0	0.0	0.0
Ascophyllum nodosum	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	13.3	19.7
	Winter									
	SGD (S1)					Control (S3)				
	Q1	Q2	Q3	Q4	Q5	Q1	Q2	Q3	Q4	Q5
Substrate	0.0	15.0	3.5	14.3	5.3	47.7	64.4	41.1	47.0	0.7
Cladophora rupestris	0.0	0.0	0.0	0.0	0.0	6.5	5.9	10.7	14.5	45.0
Fucus ceranoides	98.0	84.0	83.5	74.1	91.6	45.8	29.7	48.2	38.5	54.4
Elminius modestus	2.0	1.0	13.0	11.6	3.2	0.0	0.0	0.0	0.0	0.0

Table 8-2.4 Macroalgal biomass

	Summer									
	SGD (S1)					Control (S2)				
	Q1	Q2	Q3	Q4	Q5	Q1	Q2	Q3	Q4	Q5
Chaetomorpha linum	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Cladophora rupestris	0.04	0.01	0.00	0.00	0.00	2.93	5.07	1.26	1.67	0.72
Fucus ceranoides	35.1	90.4	55.1	79.9	32.8	9.3	14.2	17.8	13.3	1.7
Polysiphona lanosa	0.00	0.00	0.00	0.00	0.00	0.10	0.00	0.00	0.00	0.00
Ulva spp.	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	Summer									
	SGD (S1)					Control (S3)				
Polysiphonia spp.	Q1	Q2	Q3	Q4	Q5	Q1	Q2	Q3	Q4	Q5
Fucus ceranoides	177	116	149	76	124	0.05	0.10	1.29	0.00	0.00
Ulva spp.	0.00	0.00	4.52	0.00	2.60	22.2	0.0	13.7	0.0	53.4
Cladophora rupestris	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	9.36

Table 8-2.5 Macroinvertebrate abundance

	Summer									
	SGD (S1)					SGD (S2)				
	Q1	Q2	Q3	Q4	Q5	Q1	Q2	Q3	Q4	Q5
Jaera albifrons	111	98	2	74	0	141	62	74	19	138
Chaetogammarus marinus	6	5	2	20	3	12	21	20	20	6
Enchytraeus albidus	1	0	4	1	0	0	0	0	0	0
Beetle	0	1	0	0	0	0	0	0	0	0
Chironimid larvae	0	0	0	0	0	5	0	1	1	3
Carcinus maenus	0	0	0	0	0	5	0	2	0	0
Ostracoda spp.	0	0	0	0	0	10	1	11	0	0
	Winter									
	SGD (S1)					Control (S3)				
	Q1	Q2	Q3	Q4	Q5	Q1	Q2	Q3	Q4	Q5
Chaetogammarus marinus	1	7	46	4	24	0	0	0	0	2
Jaera albifrons	2324	143	418	216	1476	0	0	0	0	1
Procerodes littoralis	0	0	1		3	0	0	0	0	0
Littorina littorea	0	0	8	3	0	0	0	0	0	0
Corophium volutator	0	0	0	0	0	32	32	25	154	0
Tubifex costatus	0	0	0	0	0	0	1	25	0	1
Phyllodocidae	0	0	0	0	0	0	1	0	0	0
Neris diversicolor	0	0	0	0	0	2	1	4	2	0
Chironomid larvae	0	0	0	0	0	0	0	0	0	4
Carcinus maenus	0	0	0	0	0	0	0	0	1	0
Ostracoda spp.	0	1	0	0	0	0	0	0	0	0

Appendix C-3 Olhos de Agua (Chapter 4) raw data

Table 8-3.1 Rockpool physicochemical and morphological parameters

Rockpool	/ Control	GPS location (lat(N)/long(W))	Salinity	Fraction (%) freshwater SGD	Max. length (cm)	Perp. length (cm)	Max. depth. (cm)	Dimension (m^3)	Vol. SGD (m^3)
1	SGD	37°5'23.56"N 008°11' 9.23"W	4.27	88.1	150	97	3	0.04	0.035
2*	SGD	37°5'23.54"N 008°11' 9.59"W	15.44	57.3	210	2	57	0.02	0.012
3*	SGD	37°5'23.57"N 008°11' 9.80"W	26.22	27.4	78	223	27	0.47	0.129
4*	SGD	37°5'23.47"N 008°11' 9.60"W	29.21	19	140	225	50	1.58	0.300
5*	SGD	37°5'23.70"N 008°11' 9.92"W	31.3	13.2	530	230	27	3.29	0.434
6*	Control	37°5'23.47"N 008°11' 10.32"W	32.61	9.6	71	148	38	0.4	0.038
7	Control	37°5'23.70"N 008°11' 10.24"W	34.18	5.2	640	380	43	10.45	0.543
8*	Control	37°5'23.70"N 008°11' 10.28"W	34.53	4.4	540	240	42	5.44	0.239
9	Control	37°5'23.79"N 008°11' 9.39"W	34.6	4.1	238	206	68	3.33	0.137
10*	Control	37°5'23.63"N 008°11' 9.71"W	35.77	0.7	78	143	32	0.36	0.003
11*	Control	37°5'23.60"N 008°11' 9.51"W	36.07	0	372	173	73	4.70	0

Table 8-3.2 Rockpool nitrogen data

TON (NO_3^- + NO_2^-)		
Rockpool	ug/L	uM
1	6082	434.22
	6075	433.72
	6012	429.20
11	212	15.15
	210	15.03
	221	15.75
7	519	37.08
	521	37.19
	520	37.13
5	1347	96.16
	1350	96.42
	1346	96.09
10	202	14.43
	201	14.32
	200	14.31
3	2634	188.08
	2633	187.97
4	1849	132.01
	1832	130.82
	1816	129.68
6	1222	87.22
	1218	86.94
	1191	85.05
1	7224	515.72
	7208	514.61
	7216	515.18
9	473	33.75
	474	33.83
	471	33.65

	TON (NO_3^- + NO_2^-)		
		ug/L	uM
	Marine	375	26.80
		375	26.80
		374	26.73
	ARQ1	6560	468.57
		6310	450.71
		6050	432.14
	ARQ2	4660	332.86
		4580	327.14
		4340	310.00
		4260	304.29
	ARQ3	3730	266.43
		3570	255.00
		3520	251.43

Table 8-3.3 Rockpool percentage cover data

Rockpool	1		8		6		4		7		2		3		5	
Replicate	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
Substrate	50.28	60.87	7.64	45.14	57.62	61.82	65.52	90.97	18.07	18.62	56.97	34.84	55.70	55.86	43.02	48.61
Plocamium cartilagineum	0.00	0.72	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Cystoclonium perpureum	0.00	0.00	35.42	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Gigartina acicularis	16.95	13.04	0.00	0.00	0.00	0.00	33.10	9.03	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Gelidium spinosum	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	12.65	1.38	22.42	21.94	0.00	0.69	30.23	31.94
Dictyota dichtonia	0.00	0.00	0.00	0.00	0.66	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Phaeophyceae	0.00	0.00	0.00	0.00	13.25	3.03	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Chordaria flagelliformis	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	39.24	29.66	0.00	0.00
Ulva spp.	0.00	0.00	6.25	3.47	1.99	12.73	1.38	0.00	18.67	12.41	16.36	43.23	5.06	7.59	11.63	1.39
Ellisolandia elongata	0.00	0.00	50.69	51.39	26.49	22.42	0.00	0.00	35.54	67.59	0.00	0.00	0.00	0.00	1.74	16.67
Mytilus galloprovincialis	32.77	25.36	0.00	0.00	0.00	0.00	0.00	0.00	15.06	0.00	4.24	0.00	0.00	6.21	13.37	1.39

Appendix C-4 Kinvara bay (Chapter 5)

Table 8-4.1 Water physicochemical parameters

	Site	Salinity (psu)	pH	Temperature (°C)
Summer	GW 2011	0	7.21	16.4
	SGD 2011	0.1	6.94	11.8
		0.5	7.28	12.3
		0	6.98	11.8
		0	7.04	11.6
	SGD/marine	0	7.07	11.2
	Control (S2)	13.9	8.13	15.4
		13	8.17	15.4
		10.8	7.97	14.7
		10.8	7.94	14.3
Winter	GW	0	7.4	11.6
	SGD(S1)	0	7.18	11.3
		0	7.41	11.4
	Galway Bay	33	8.2	10

Table 8-4.2 Water nitrogen data N as nitrate and nitrite (NO3 + NO2)

Summer		
Site	ug/L	µM
GW 2011	917	65.5
	963	68.79
	921	65.79
SGD 2011	884	63.14
	827	59.07
	818	58.43
	816	58.29
SGD/marine	797	56.93
	903	64.5
	890	63.57
	850	60.71
	860	61.43
SGD/Marine	373	26.64
	424	30.29
	434	31
	400	28.57
	408	29.14
Control (S2)	216	15.43
	218	15.57
	222	15.86
	219	15.64

Winter		
Site	ug/L	µM
SGD 2012	1590	113.57
	1400	100
	1290	92.14
	1150	82.14
	1120	80
	1130	80.71
	1250	89.29
	1250	89.29
	1240	88.57
Galway bay	145	10.36
	141	10.07
GW 2012	840	60
	841	60.07
	846	60.43

Table 8-4.3 Water isotopic composition

Site	δ^{15}N vs. Air	δ^{18}O vs. V-SMOW
SGD (S1)	8.61	16.78
	8.54	16.52
SGD/Control	8.15	17.88
	8.15	18.10
Control (S2)	6.68	18.59
	6.59	18.38
Control /Marine	6.30	17.37
	6.35	17.58
Groundwater 2011	7.95	16.38

Table 8-4.4 Algal isotopic composition and elemental data for summer sampling campaign

2011 (summer)											
SGD (S1)						Control (S2)					
Species	C%	$\delta^{13}C$	N%	$\delta^{15}N$	C/N	Species	C%	$\delta^{13}C$	N%	$\delta^{15}N$	C/N
U. linza	37.93	-32.14	5.00	5.51	8.86	*U. linza*	35.03	-22.86	3.47	6.19	11.77
U. linza	37.73	-31.87	4.88	5.73	9.02	*U. linza*	35.61	-22.59	3.83	6.39	10.83
F. ceranoides	41.65	-26.92	2.10	5.93	23.11	*U. linza*	36.16	-27.93	4.00	4.56	10.54
F. ceranoides	37.54	-26.40	2.10	6.95	20.83	*U. linza*	35.32	-27.76	3.88	4.88	10.62
F. ceranoides	36.85	-25.52	2.01	6.86	21.40	*U. linza*	36.30	-25.74	3.23	5.96	13.09
F. ceranoides	42.49	-30.04	2.49	5.74	19.94	*F. ceranoides*	36.85	-20.52	2.02	6.13	21.27
F. ceranoides	38.25	-32.91	2.11	5.93	21.09	*F. ceranoides*	36.18	-21.52	2.01	6.03	20.99
F. ceranoides	38.43	-31.11	2.15	5.85	20.88	*F. ceranoides*	38.01	-22.21	2.07	6.35	21.42
F. ceranoides	41.03	-26.23	2.25	6.04	21.31	*F. ceranoides*	36.19	-21.67	1.83	5.66	23.11
F. ceranoides	36.91	-33.55	2.10	7.01	20.47	*F. ceranoides*	37.67	-27.36	2.41	5.40	18.21
F. ceranoides	37.87	-34.15	2.49	6.69	17.76	*F. ceranoides*	37.89	-26.92	2.22	6.60	19.90
F. ceranoides	36.83	-28.91	2.19	8.36	19.59	*F. ceranoides*	39.30	-22.92	2.21	6.14	20.72
F. ceranoides	37.86	-33.97	2.28	8.65	19.33	*F. ceranoides*	38.76	-22.97	2.10	6.31	21.49
F. ceranoides	33.41	-29.92	2.41	5.63	16.15	*F. ceranoides*	38.12	-25.98	2.27	6.56	19.61
F. ceranoides	40.36	-33.99	2.46	6.39	19.13	*F. ceranoides*	38.70	-22.71	2.08	5.91	21.71
F. ceranoides	39.30	-35.36	2.32	6.33	19.76	*F. ceranoides*	38.82	-22.53	2.05	6.01	22.04
F. ceranoides	38.40	-30.81	2.58	6.47	18.66	*F. ceranoides*	37.95	-22.28	2.27	5.74	19.47
						F. ceranoides	38.41	-21.73	1.98	5.99	22.63
						F. ceranoides	38.56	-21.90	2.01	6.05	22.42

Table 8-4.5 Algal isotopic composition for winter sampling campaign

Winter (2012)						
SGD (S1)				Control (S3)		
	$\delta^{15}N$	$\delta^{13}C$			$\delta^{15}N$	$\delta^{13}C$
F. ceranoides	6.985	-34.6088		*F. ceranoides*	9.82	-26.1835
F. ceranoides	7.8967	-29.1119		*F. ceranoides*	6.92	-25.3189
F. ceranoides	9.1278	-32.2794		*F. ceranoides*	6.36	-25.91
F. ceranoides	8.5245	-41.8603		*F. ceranoides*	7.60	-26.9735
F. ceranoides	7.0978	-36.6933		*F. ceranoides*	8.97	-26.1735
F. ceranoides	7.5031	-33.4307		*F. ceranoides*	8.51	-27.1437
F. ceranoides	7.1388	-41.9216		*U. intestinalis*	6.39	-25.0328
U. intestinalis	6.0096	-39.7393				
U. intestinalis	5.922	-43.3969				

Table 8-4.6 Macroinvertebrate isotopic composition and elemental data for the summer dataset

2011 (Summer)						
Species	Site	C%	$\delta^{13}C$	N%	$\delta^{15}N$	C/N
J. albifrons	SGD (S1)	26.06	-29.74	5.64	8.44	5.39
J. albifrons		24.86	-28.76	5.14	8.44	5.64
J. albifrons		22.33	-29.21	4.82	8.51	5.41
J. albifrons	Control (S2)	21.60	-24.48	5.28	9.14	4.77
J. albifrons		23.82	-24.44	5.78	9.34	4.80
J. albifrons		18.08	-23.80	4.57	8.80	4.62

Table 8-4.7 Macroinvertebrate isotopic composition for the summer data set

2012 (Winter)			
Species	Site	$\delta^{15}N$	$\delta^{13}C$
C. marinus	S1 (imp)	9.94	-28.63
C. marinus		9.67	-32.31
J. albifrons		-	-32.81
J. albifrons		10.80	-34.25
J. albifrons		9.81	-32.41
J. albifrons		10.47	-33.56
J. albifrons		10.47	-33.28
C. gammarus	S2 (control)	8.88	-24.47
C. volutator		8.48	-26.50
C. volutator		8.01	-25.39
C. volutator		9.70	-25.74
C. volutator		9.57	-25.87

Appendix D - nmMDS ordinations

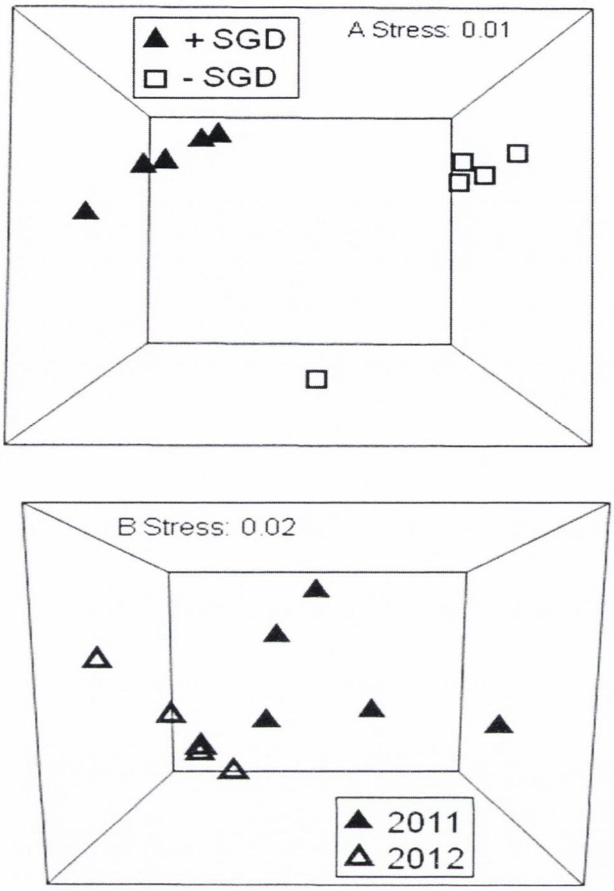

Figure 8-11.1 3D nmMDS plot of percentage cover data comparing (A) winter site 1 and control site 3 data, and (B) SGD site 1 in 2011 and 2012. All data square root (√) transformed data.

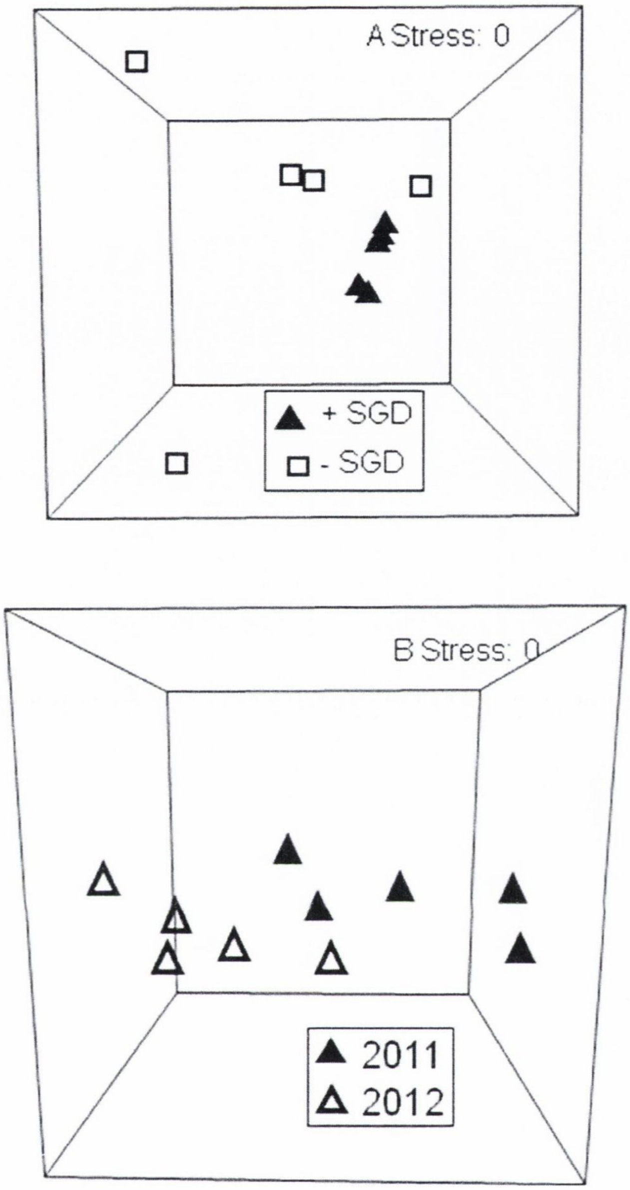

Figure 8-11.2 3D nmMDS plot of macroalgal biomass data comparing (A) winter site 1 and control site 3 data, and (B) SGD site 1 in 2011 and 2012. All data fourth root (√√) transformed data.

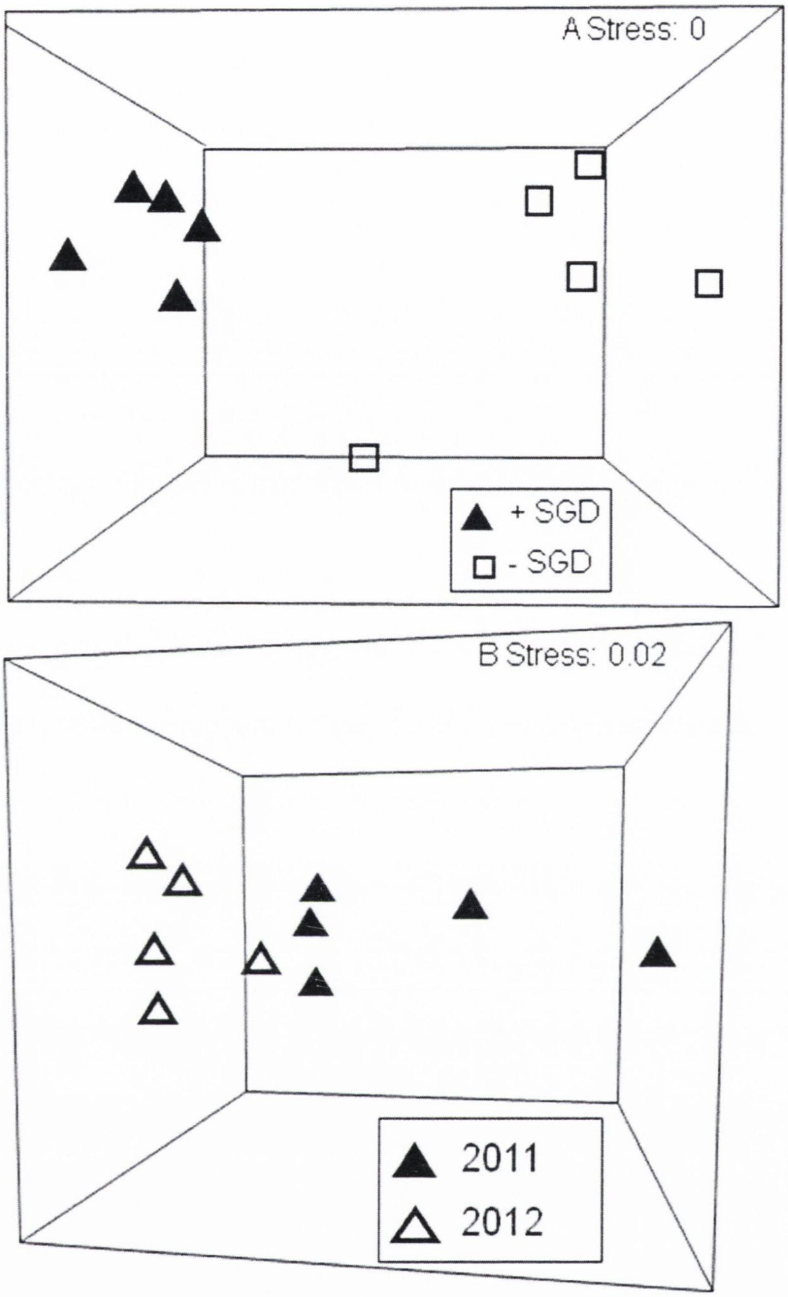

Figure 8-11.3 3D nmMDS plot of macroinvertebrate species abundance data comparing (A) winter site 1 and control site 3 data, and (B) SGD site 1 in 2011 and 2012. All data fourth root (√√) transformed data.

Appendix E - Summary table outlining *n* for each quadrat, and each site (total) (Chapter 5)

Table 8-5.1 Outline of *n* derivation for each quadrat, and each site (total)

	Summer												Winter											
	SGD (Site 1)					Total	Control (Site 2)					Total	SGD (Site 1)					Total	Control (Site 3)					Total
Quadrat	1	2	3	4	5		1	2	3	4	5		1	2	3	4	5		1	2	3	4	5	
F. ceranoides	3	3	3	3	2	14	3	3	3	3	2	14	1	1	1	1	3	7	1		1	1	3	6
***Ulva* spp.**	1	1				2	1	1	1	1	1	5	1	1				2	1					1
Site total						16						19						9						7

In summer, three replicate samples of *Fucus ceranoides* were sampled from each of four quadrats, and two replicates of *Fucus ceranoides* sampled from the fifth quadrat at both site 1 and site 2 (i.e. n = 14). All biomass of *Ulva* spp. contained a quadrat was harvested and pooled to give one *Ulva* spp. sample per quadrat; this amounted to two samples for site 1 and five samples for site 2. Therefore, for algal isotopic analysis n = 16 for site 1 and n = 19 for site 2 in summer. In winter, three replicate samples of *F. ceranoides* were sampled from each quadrat and two samples of *Ulva* spp. from site 1 and one sample from site 3. For *F.ceranoides*, for four quadrats, the three replicate samples were pooled for isotopic analysis, and for the fifth quadrat, all three samples were analysed. The composite *F. ceranoides* sample from quadrat two in site 3 was lost during processing. Therefore, for winter algal isotopic analysis, n = 9 for site 1 and n = 7 for site 3.

Appendix F - Samples size calculations

Table 8-6.1 Full details of calculations of sample size. Sample size (*n*) explanatory table, chapter 5. The calculated sample sizes (*n*) for the given desired detectable difference (*δ*) in population means.

	Macroalgae		Macroinvertebrate	
	$\delta^{15}N$	$\delta^{13}C$	$\delta^{15}N$	$\delta^{13}C$
$(Z\alpha + Z\beta)^{2}$	13	13	13	13
$2\sigma^{2}$	2	25	2	8
$((Z\alpha + Z\beta)\ 2\ )2\sigma^{2}$	26	318	26	104
δ^{2}	0	25	0	25
n	104	13	104	4
	Macroalgae		Macroinvertebrate	
	$\delta^{15}N$	$\delta^{13}C$	$\delta^{15}N$	$\delta^{13}C$
$(Z\alpha + Z\beta)^{2}$	13	13	13	13
$2\sigma^{2}$	2	25	2	8
$((Z\alpha + Z\beta)\ 2\ )2\sigma^{2}$	26	318	26	104
δ^{2}	1	49	1	49
n	26	6	26	2