Biodiversity of freshwater sponges (Porifera: Spongillidae) in the UK: taxonomy, distribution, and ecology.

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Abstract

Specimens of freshwater sponges (Porifera: Spongillidae), one of the most poorly known faunal groups in the UK, were obtained from natural (lentic and lotic) habitats and from anthropogenic (canal) habitats. The investigations encorporated a multidisciplinary approach to address questions of species diversity, species distribution, abundance, key water parameters, spicule variability, sequence divergence, and molecular and morphological taxonomy. A total of six species of sponges were identified (*Spongilla lacustris*, *Ephydatia fluviatilis*, *Ephydatia mülleri*, *Eunapius fragilis*, *Racekiela ryderii*) including a species new to the UK: *Trochospongilla horrida*. The morphology, taxonomy, distribution, and ecology of each species were examined. A taxonomic key to UK species of freshwater sponges was presented. New limits of tolerance to water parameters were established for species. An analysis of a partial COI mtDNA gene sequences was conducted to determine variation within and between freshwater sponge species, and was followed with an analysis of marine species. The resolution power of the COI gene was compared with 28S rDNA and ITS rDNA sequence data from UK freshwater sponge species. Low variability of COI gene sequences was found, with low nucleotide diversity, suggesting a high conservation of mtDNA in sponges. Populations of a common and widely distributed species (*Spongilla lacustris*) were investigated to assess increased spicule size in habitats with low dissolved phosphorus and increased pH. Populations of a rare UK species, with an amphiatlantic distribution (*Racekiela ryderii*) were investigated to assess spicule variability and evidence of a 'form' called *pictovensis*. However, insufficient genetic variability in COI and ITS genes were observed within this species supporting the inclusion of the *pictovensis* form into the species *R. ryderii*. An integrated approach using genetic and morphological characteristics to identify sponge species identification was suggested. Both genetic sequence data together with morphology provided valuable taxonomic insights and contributed to the formation of an integrated picture of biodiversity in natural and anthropogenic UK freshwater habitats.

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Preface

The structure of this thesis follows recommendations made by Stanisstreet (1996). It is a sequential series of linked chapters, bracketed by a general introduction and general conclusion. Key questions and aims are outlined in the General Introduction. Self-contained investigations that follow from these aims are outlined in each chapter. Each chapter was conceived as a paper that follows the style of Freshwater Biology, an appropriate target journal for this subject matter. For this reason, some sections (e.g. Methods) are repetitive. One advantage of the chosen structure is that it facilitates the preparation of sections of the work for publication (Stanisstreet, 1996).

Stanisstreet M (1996) Writing your thesis. Suggestions for planning and writing theses and dissertations in science-based disciplines. The University of Liverpool, 14 pp.

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Chapter 1. General Introduction: Biodiversity of UK freshwater sponges (Porifera: Spongillidae).

Freshwater biodiversity

The term biodiversity incorporates not just species diversity and the genetic differences between species, but also the habitat diversity, in which species occur (Reynolds and Souty-Grosset, 2011). Significant declines in biodiversity are predicted by the middle of this century (Jenkins, 2003). Although the overall number of freshwater species is lower than in marine or terrestrial systems, extinction rates for freshwater fauna are projected to be greater than in these systems (UNESCO, 2003; Ricciardi and Rasmussen, 1999; Dudgeon *et al*., 2006). Freshwater organisms are threatened globally because their habitats (inland waters) are central to economic sustainability, and human demands for water are expanding (Reynolds and Souty-Grosset, 2011). Freshwater biodiversity is especially vulnerable to human activities because of the disproportionate species richness (6% of species) living in inland waters (0.01% of the world's water) (Dudgeon *et al*., 2006; Reynolds and Souty-Grosset, 2011). Data are insufficient to estimate accurately loss rates of freshwater biodiversity in many regions (Dudgeon *et al*., 2006), but extinction rates of freshwater animals in North America have been estimated as 4% decade^{-1} (Ricciardi and Rasmussen, 1999).

There has been no comprehensive analysis of freshwater biodiversity comparable to those completed for terrestrial systems (Dudgeon *et al*., 2006). In the UK, the total biodiversity of freshwater ecosystems remains to be determined, and is particularly incomplete among invertebrate taxa (Dudgeon *et al*., 2006). This means that even with predictable reductions in overall species numbers, forecasting the identities of the affected taxa is not possible. Approaches to assess biodiversity applied to other ecosystems (e.g. terrestrial ecosystems) may not accurately reflect estimates of UK freshwater biodiversity (Dudgeon *et al*., 2006). One approach to quantifying species biodiversity is the study of the genetic structure by measuring sequence diversity of certain genetic markers (Müller *et al.,* 2003). Biodiversity is closely correlated with genetic diversity, since the diversity of the sets of genes present in a given taxon has to be considered as (1) a basis for its evolutionary

potential and (2) resources for a changing environment (Müller *et al.,* 2003). Investigating patterns of freshwater invertebrate diversity and assessing possible mechanisms of diversity could provide a baseline for future predictions of change.

The overall aims of this PhD were to assess patterns of freshwater invertebrate diversity and assessing possible drivers of diversity. Firstly species richness and patterns for UK taxa were investigated. As species abundance as well as species richness is a component of diversity, the second aim was to assessing the water parameters that are drivers of species abundance. Thirdly, biodiversity assessments were taken a step further by evaluating species identification methods and assessing genetic diversity between and within groups. And lastly (fourth and fifth aims), as focusing on individual species is a valuable means of monitoring biodiversity (Reynolds and Souty-Grosset, 2011), investigations were carried out contrasting one common and one rare species in terms of morphological and genetic diversity within each species.

Using genetic diversity to assess biodiversity is useful for freshwater invertebrates (Dudgeon *et al*., 2006), especially with invertebrate taxa that are understudied, have complicated life cycles, or possess comparatively few easily identifiable morphological characters in the adult stage (Sole-Cava *et al*. 1992). Such taxa include the sessile freshwater invertebrates, sponges, bryozoans, and cnidarians. These groups are particularly important as they are often difficult to identify (Reiswig *et al*., 2009) and consequently are ignored in some diversity assessments (Ricciardi, 1992). In addition they are directly affected by local habitat parameters (Müller *et al.,* 2003) and may have restricted ranges (Dudgeon *et al*., 2006). The present study aims to increase the knowledge of an understudied freshwater invertebrate group, specifically sponges (Porifera). Investigating patterns of freshwater sponge diversity and assessing possible mechanisms of that diversity could provide a baseline from which predictions of future change can be made.

Sponges

Focusing on sponges (phylum Porifera) as a diverse group of primitive metazoans, largely unchanged in their fundamental "bauplans" since the Late

Cambrian (509 mya), permitted assessment of distribution patterns and mechanisms of biodiversity. Freshwater sponge species are not often considered charismatic and their importance is not well recognized (Reynolds and Souty-Grosset, 2011). Sponges exist in their present form as a result of their ecology, and their evolution (Müller *et al.,* 2003). The basic organization of a sponge consists of a dermal epithelium, composed of pinacocytes, surrounding an organic matrix (mesohyl) containing specialized cells including choanocytes, and archaeocytes. Cells within the mesohyl interact with the pinacocytes in gas exchange, digestion, structural support, and reproduction. In the UK freshwater sponge form undergoes a life-cycle involving gemmule hatching (Fig. 4f, g) and formation of functional cells (e.g. choanocytes, pinacocytes, archaeocytes) in the spring, prolific growth phase including sexual reproduction during the summer, and asexual reproduction with the degeneration or death of adult tissue in the autumn (Simpson and Gilbert, 1973; Jewell, 1939; Harrison, 1974; Poirrier, 1974; Van de Vyver and Willenz, 1975; Bergquist, 1978; Williamson and Williamson, 1978) (Fig. 4).

Importantly, freshwater sponges are filter feeders and with their arrangement of inhalant pores and choanocytes place a series of sieves in the path of particles in a water current (Bergquist, 1978). Chambers with choanocyte are the keystones of the sponge feeding system. Each chamber consists of numerous choanocytes that are characterized by the presence of a flagellum and a collar of microvilli. Beating by the flagella within these chambers is primarily responsible for setting up the flow of water through the sponge. Choanocytes serve as the final filters in the feeding system. Using this method, sponges have the ability of filtering smaller particles than do other filter feeders (<5 μm) (Frost, 1978, 1980; Pile *et al*., 1996). Through these filtering activities, freshwater sponges are capable of processing vast quantities of water (Reiswig, 1971; Vogel, 1974) with estimates of a sponge size 10cm long filtering >125 L day^{-1} (Reiswig *et al*., 2009). Some marine sponges have been estimated to filter the entire volume of water in their communities every 24-48 hours (Reiswig, 1971; Savarese *et al*., 1997). The freshwater sponge *Spongilla lacustris* has a filtration and clearance rate of 0.24 ml sec^{-1} (per ml sponge) (Frost, 1980). Following filtration, particles are phagocytosis by choanocytes, quickly processed to the base of the cell, and then transferred to archaeocytes (Willenz, 1980). Food

processing occurs in archaeocytes together with nutrient storage (Simpson, 1984), so each individual archaeocyte can be considered as a separately functioning digestive unit with the ability to vary its actions with the characteristics of the food particle (Frost, 1980). Archaeocytes move freely in sponge mesohyl and are able to transfer digested materials to other cells, such as those involved in skeleton formation. Non-digested materials are released into the water flowing in exhalent passages, or to the exterior by a reverse phagocytic action (Bergquist, 1978) and are carried out through excurrent passages (Frost, 1980).

An additnal nutritional path involves particles adhering to the sponge dermal layer. The sponge dermal layer is formed from pinacocytes and has a dual role of gathering particles by adherence together with internalizing particles by endocytosis. Adherence of particles depends on their biochemical and morphological properties. Development of cytoplasmic folds and endocytosis suggests a high metabolic activity in exopinacocytes, and is followed by transit to endopinacocytes (Willenz, 1980). Particle phagocytosis occurs in pinacocytes in all areas of the sponge (Willenz, 1980; Willenz and Van de Vyver, 1982); even pinacocytes acting to attach the sponge to its substrate actively ingest particles (Harrison, 1972). Bergquist (1978) suggest that archaeocytes close to pinacocytes lining inhalent passages form an important capture system, with phagocytosis occurring as particles come into contact with the passage wall. Particle removal by phagocytosis in pinacocytes could, theoretically, occur at the same time as filtering by choanocytes, particularly when species have a relatively flat growth form with short inhalent passages. In this way, the capacity of sponges to remove suspended particles from the water column (Reiswig *et al*., 2009; Frost, 1978, 1980), contributes to both water quality and nutrient cycling (Reiswig *et al.,* 2009).

Freshwater sponges have additional ecological roles as they are preyed upon by other animals including ducks (McAuley and Longcore, 1988), crayfish (Williamson, 1979), spongillaflies (Resh, 1976), snails (Reiswig *et al*., 2009), and rotifers (Bērzinš, 1950). Various taxonomic groups (Oligochaetes, Leeches, Bryozoa and Nematodes) have been found on or within freshwater sponges, although few of these animals, have a direct relationship with the sponge (Matteson, and Jacobi,

1980; Traveset, 1990). However, an adult sponge can provide a refuge for animals e.g. mites (Unionicolidae) which lay eggs inside a sponge (Procter and Harvey, 1998), and the bivalve *Eupera* (Volkmer-Ribeiro and De Rosa-Barbosa, 1974). However, although freshwater sponges are reported to be widely distributed (Penney and Racek, 1968), freshwater diversity assessments have not been undertaken.

Assessing one or a few water bodies cannot reveal a significant proportion of freshwater biodiversity within a region (Dudgeon *et al*., 2006). To obtain overall UK distribution patterns, data from a wide range of freshwater habitats as possible was obtained. This was achieved in a number of ways including, i) visiting >200 UK sites ranging from Scotland to southern England, and Wales, ii) visiting the Natural History Museum, London to view archive material and to borrow selected samples, iii) collaboration with the Upland Waters Monitoring Network (Environmental Change Research Centre, UCL), iv) contacting >80 marinas and boat mechanics, v) launching a web based campaign to ask for samples, vi) sending sampling kits to selected individuals and organisations, and vii) conducting a thorough literature search of historic records (Table 1). In these data a broad survey indicating species distributional patterns in UK freshwater habitats could be obtained. This survey included natural lentic (Fig. 1a, b, c) and lotic habitats (Fig. 1d, e, f). Research then focused on sponge populations in targeted canal waterways (Fig. 2) with both natural and anthropogenic inputs. This was important as canal characteristics, such as variable water parameters, differing degrees of waterway connectivity, and easily accessible substrate enabled a thorough choice of sampling sites to pursue assessments into species diversity.

Diversity drivers within canal habitats

Sponges, together with other invertebrates, play multiple roles in the functioning of freshwater habitats (Dudgeon *et al*., 2006). However, little is known about how water parameters influence sponge species diversity or UK distribution. Canal characteristics allowed the relative importance of variation in water parameters to be investigated with regard to mechanisms of diversity, specifically freshwater sponge species presence, population abundance, and morphological variation. Following the start of the industrial revolution in 1760, approximately 3000km of

canals were built (Fig. 3a), connecting rivers (Mersey, Trent, Severn, Thames), in proximity to other freshwater habitats (Fig. 3b), and thus effectively linking previously fragmented areas (Yorke, 2008). Subsequently, from 1870 canal transport was superseded by other transport methods. Neglect and deterioration of some canals resulted in isolated stretches with lower anthropogenic input (Willby and Eaton, 1996). The present study targeted canals in the north-west of the UK, as representative sampling sites of UK anthropogenic waterways. For example, the Shropshire Union Canal (Fig. 3) was included in the investigation as it consists of a long (>300km) branched network, it moves through both industrial and agricultural surroundings (Fig 2b), runs from a port (Ellesmere Port) south to the Midlands (Wolverhampton) (Fig. 3a), has extensive boat traffic (Yorke, 2008), and contains a Site of Special Scientific Interest. Additionally, following a breach in 1930s, one section was abandoned and drained (Hadfield, 1959) (Fig. 2g), so it contains both connected and isolated sections. Thus, these targeted canals encompassed the complex nature of freshwater habitats, with varying connectivity and spatial heterogeneity (Reynolds and Souty-Grosset, 2011).

Variability of water parameters (Thorpe and Covich, 2009; Pronzato and Manconi, 1994) is an important factor in determining the mechanisms of species distribution. Specific parameters were targeted because of their possible effect on species distribution (see Chapter 3). Several previous studies have investigated the presence of sponges with different water chemical properties i.e. temperature, conductivity, pH, total dissolved solids, phosphate, silicate, calcium, as summarised by Harrison (1974). In addition oxygen content, oxidation reduction potential, and salinity have been recorded in other, more recent, studies (Ricciardi and Reiswig, 1993; Williams and Fulthorpe, 2003). Variations in some parameters are seasonal (e.g. water temperature recorded as part of pilot study, Supporting Information 1.1, Fig. S2) and others (e.g. phosphorus), can be attributed to canals passing through different catchment areas (Bishop, 1995; Neal *et al*., 2005) or from pollution (Fig 2h). Canal habitat features allow the relative importance of variation in water parameters to be investigated with regard to distribution patterns of freshwater sponge species.

Present knowledge, sampling effort, and species identification methods

As species richness is a major component of biodiversity (Reynolds and Souty-Grosset, 2011), accurate taxonomic information was required to determine freshwater sponge species present in a habitat. There are approximately 260 species of freshwater sponges worldwide (van Soest *et al*., 2015; Manconi and Pronzato, 2008), that represent about 3% of all diversity in Porifera. Investigations to determine species richness of freshwater sponges have been conducted in some European countries (Table 2). Whereas in the UK, fragmentary sponge records (Table 1) relate to a single habitat or a limited number of microhabitats. This lack of information is important because a fragmented collection of reports may not reflect the true levels of freshwater habitat biodiversity. A summary of such records (see Pronzato and Manconi, 2001) has identified five UK freshwater sponge species:

Phylum: Porifera Grant, 1836
- Class: Demospongiae Sollas, 1885
  - Subclass: Heteroscleromorpha Cárdenas, Perez and Boury-Esnault, 2012
    - Order: Spongillida Manconi and Pronzato, 2002
      - Family: Spongillidae Gray, 1867
        - *Ephydatia fluviatilis* (Linnaeus, 1759)
        - *Ephydatia müller*i (Lieberkühn, 1856)
        - *Eunapius fragilis* (Leidy, 1851)
        - *Racekiela ryderii* (Potts, 1882)
        - *Spongilla lacustris* (Linnaeus, 1759)

Together with species richness, biodiversity also includes species evenness, and rarity (Reynolds and Souty-Grosset, 2011). Thus, it was important to determine that if sponge species were not found at a sampling site it was because they were not there and not because they had not been detected during sampling. Therefore the sampling effort to determine all species at a site was considered (Supporting Information 1.1, Box S1) and it was determined that all species present within a site could be collected within half an hour. This investigation into sampling effort was carried out at a variety of sites as a pilot study to determine the level of sampling required to make an unbiased estimate of species richness (for targeted sampling) and underlies the methods used in thesis chapters 2,3, and 5.

Species richness estimates require accurate identification methods. This involved a multi-disciplinary approach using both morphological taxonomic and molecular methods for data in all chapters and especially Chapter 4. Observations used to identify sponges (Chapter 2) included gross morphology and microscopic morphology. However, using gross morphology (Fig. 4) to identify sponges was problematic because of the highly variable nature of external appearance (see Bidder, 1923; Palumbi 1984 Manconi and Pronzato, 1991). For example, some colonies of freshwater sponge possess symbiotic zoochlorellae resulting in a green coloured colony (Fig. 4b, e) (Simpson, 1984; Reiswig *et al*., 2009). Also, morphological changes occur throughout the annual life cycle of freshwater sponges (Supporting Information 1.1, Fig. S2), alternating between predominately adult sponge tissue (e.g. Fig. 4a, b) or predominately gemmules (Simpson and Gilbert, 1973; Van de Vyver and Willenz, 1975; Bergquist, 1978; Williamson and Williamson, 1978), (Fig. 4c, d, e).

Instead sponges were identified from characteristic components of the sponge skeleton (see an outline of sponge morphology in Bergquist, 1978), which in Demospongiae are composed of hydrated, amorphous silica (Simpson, 1984). Freshwater sponge skeletal components are silica spicules bound together with collagen. Size and form of the siliceous spicules represent the prominent taxonomic character. The spicules are grouped into the smaller microscleres and the larger megascleres, and their shape and size are traditionally used as part of the diagnostic characters in sponge taxonomy (e.g. Hooper and van Soest, 2002). Megascleres are produced by all actively growing sponges (Fig. 4f, g). Length is species specific and falls within a range of 100 to 450µm (Reiswig *et al*., 2009). Microscleres are skeletal elements that are widely distributed throughout the body of only some sponge species (e.g. *S. lacustris*) thus their presence/absence was useful in identification. In addition to skeletal spicules, sponge classification relies heavily on the features of gemmules and gemmuloscleres (Fig. 4d) (Reiswig *et al*., 2009; Penney and Racek, 1968; Manconi and Pronzato, 2002).

Problems in freshwater sponge species identification arise when gemmules were absent, and the other morphological characteristics were inadequate. To

overcome the problem of identifying sponge species molecular data have become widely used. Allozymes, alternative forms of enzymes, were the first molecular markers used as tools for improved taxonomic resolution in marine invertebrates (Thorpe and Solé-Cava, 1994). Following these early developments there have been attempts to standardise the protocols for species determination. More recently, DNA barcoding, the use of a single, standardised genetic locus to delineate species across different taxa was suggested by Hebert *et al*. (2003a, b). This technique, subsequently has been used to assess biodiversity in invertebrate taxa e.g. butterflies (Hebert *et al*., 2004b) and moths (Mutanen *et al*., 2013).

In DNA barcoding, gene sequences function as species-specific signature sequences (i.e. DNA barcodes) that allow species identification (Hebert *et al*., 2003a, b). The typical DNA barcoding locus is based on a mitochondrial cytochrome c oxidase subunit 1 (COI) gene sequence. DNA barcoding relies on the feature that, for many species, the level of interspecific variation at COI is greater than intraspecific variation resulting in a distinct gap in the distribution of sequence variation, or the 'barcoding gap', that allows species to be identified (Hebert *et al*., 2003a, b; Shearer and Coffroth, 2008). Values of interspecific COI sequence divergence are usually greater than 2−3% (Hebert *et al*., 2003a, b; 2004a, b), whereas variation within members of the same species (intraspecific) is often less than 1% (Hebert *et al*., 2004a). While there is an apparent 'barcoding gap' for some invertebrate taxa (e.g. moths, Hebert *et al*., 2004b; spiders, Barrett and Hebert, 2005), a low mean COI sequence divergence (≤1%) has been observed among other invertebrate taxa (e.g. cnidarians, Shearer and Coffroth, 2008). Freshwater sponge identification was assessed using this method and from the use of other genetic markers (nuclear DNA genes) such as 28S rDNA, and ITS2 rDNA sequences (see Addis and Peterson, 2005; Lopp *et al*., 2007).

Research aims

The overall aims of this PhD have been outlined. More specifically, freshwater invertebrate distribution patterns using sponges as a model organism were investigated by assessments of species presence, abundance, and genetic diversity. A series of testable hypotheses were proposed with the following aims:

i. To compile a species list, identify species distribution, and describe the freshwater sponges which occur in the UK as without knowing the current distributions of these native invertebrates it is not possible to identify changes in community composition over time (Chapter 2). This involved species identification using morphological methods and supported by a molecular approach, based on sequencing a section of 28S rDNA.

ii. To provide fundamental knowledge to facilitate the use of freshwater sponges in diversity studies and determine factors influencing patterns of freshwater sponge species distribution and abundance in canal habitats (Chapters 2 and 3). The overall goal was to provide summer demarcation values of important water parameters that affect sponge abundance in canal habitats, contrasting the effects of seasonal parameter variability, species requirements, and canal ecology.

iii. To identifying sponge diversity using spicule morphology and COI barcoding from freshwater species, and compare with marine sequence data (Chapter 4). The reliability of species identification using spicule morphology was assessed. The study then quantified COI sequence divergence between pairs of species freshwater sponges collected in UK habitats. A similar genetic evaluation of COI sequence divergence in marine sponge sequences from GenBank was included to examine intra- and interspecific variabilities for sponge diversity.

iv. To analyse the mechanisms of diversity of a common UK freshwater sponge population (*S. lacustris*) using morphological methods and specific genetic characteristics (Chapter 5). The overall goal of this study was to assess variation in the size of *S. lacustris* megascleres and microscleres, and then relate how spicule morphology varied in relation to water parameters in canal habitats. The degree of population genetic variability (28S rDNA and ITS genes) was investigated.

v. To analyse the mechanisms of diversity of a rare UK freshwater sponge population (*R. ryderii*) (Chapter 6). This study provided characters that allow the recognition of *R. ryderii* in the UK, by examining spicule characteristics, and sequences of both COI and ITS genes. The overall goal was to assess *R. ryderii* distribution, and establish a morphological and genetic baseline from which to extend the assessment of this species.

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Table 1. Published records of freshwater sponges at UK sites from 1868 to 2012 using species names as they appear in the reference.

Species name in reference	Location	Reference
Ephydatia meyenii	Salmon Pool, Exeter	Parfitt, 1868
Spongilla meyeni	River Exe	Carter, 1868
Spongilla lacustris *Ephydatia fluviatilis* *Ephydatia muelleri* *Spongilla fragilis* *Tubella pennsylvanica*	Glasgow canal River Dee Loch Baa	Annandale, 1908
Spongilla lacustris *Ephydatia fluviatilis*	General	Mellanby, 1953
Ephydatia fluviatilis *Euspongilla lacustris* *Heteromeyenia ryderi*	Lochs Druidibeg, and Roag. Howmore River	Waterston and Lyster, 1979; Waterston, 1981
Spongilla lacustris, *Ephydatia fluviatilis* *Ephydatia muelleri*	River Thames	Didžiulis, 2012

Table 2. Fresh water sponge species richness in European countries in order of proximity to the UK, including: Ireland (Stephens, 1920, Lucey, and Cocchiglia, 2014), France (Pronzato and Manconi, 2001); Belgium (Richelle-Maurer *et al*., 1994), Netherlands (van Soest, 1977), Germany (Gugel, 1999, Pronzato and Manconi, 2001), Denmark (Tendal, 1967), Norway (Økland and Økland, 1996), Spain (Traveset, 1990), Poland (Pronzato and Manconi, 2001), Lithuania (Pronzato and Manconi, 2001), Latvia (Pronzato and Manconi, 2001) and Estonia (Lopp *et al*., 2007).

	S. lacustris	*E. fluviatilis*	*E. milleri*	*Eu.. fragilis*	*Eu.. carteri*	*Trochospongilla. horrida*	*Racekiela ryderi*	*Heteromeyenia stepanowii*	*H. baileyi*	*Radiospongilla cerebellata*
Ireland	x	x	x	x			x			
France	x	x	x	x		x				
Belgium	x	x	x	x		x				
Netherlands	x	x	x	x						
Germany	x	x	x	x	x	x		x	x	x
Denmark	x	x	x	x						
Norway	x	x	x	x			x			
Spain	x	x	x						x	
Poland	x	x	x	x	x	x		x	x	
Lithuania	x	x		x						
Latvia	x	x	x	x						
Estonia	x	x	x	x						

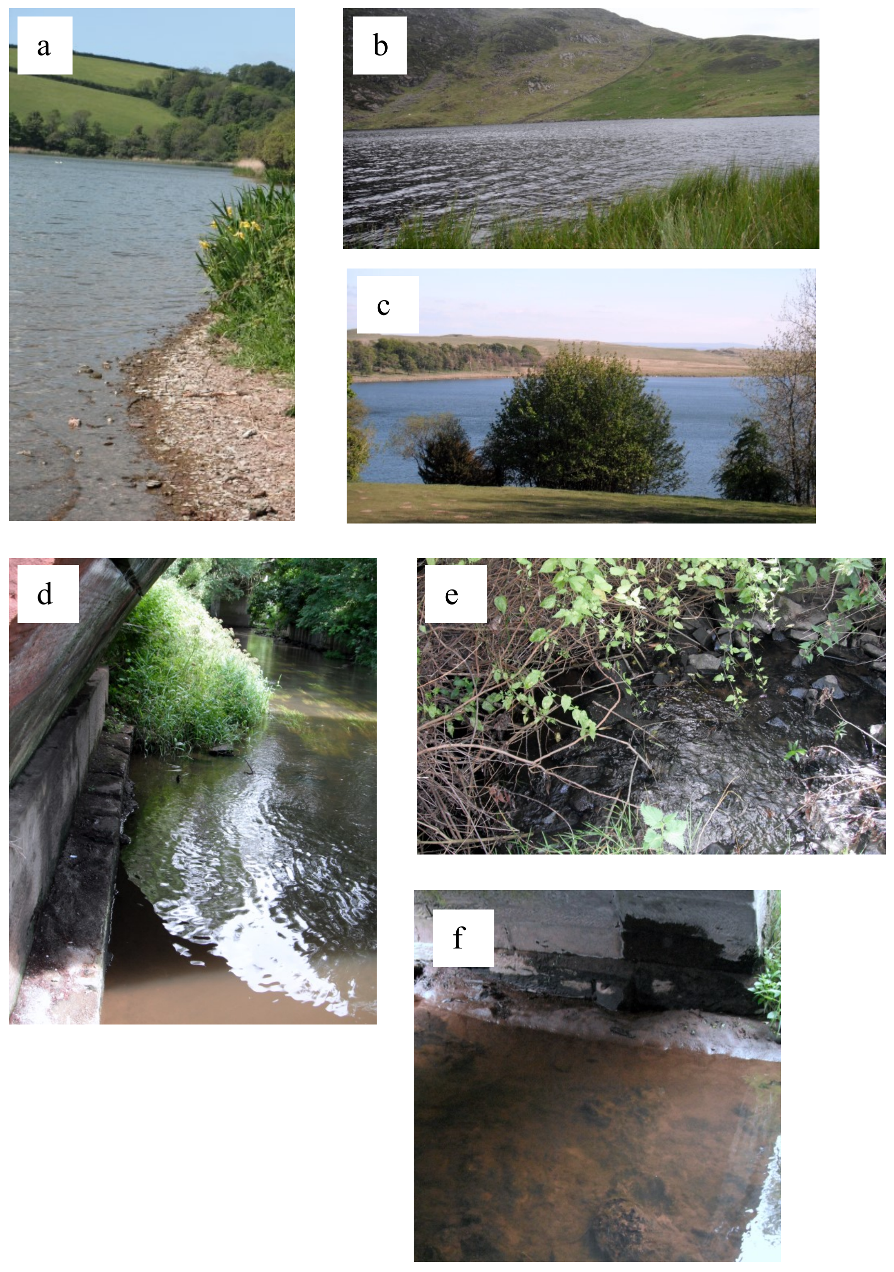


Fig. 1. Examples of lentic (a-c) and lotic (d-f) habitats examined for sponges a) Slapton Ley b) Llyn Llagi c) Malham Tarn d) view through vegetation over Eaton Brook e) slow moving, shallow water in Ledwyche Brook under a bridge f) shaded area of River Roden under a bridge.

Fig. 2. Examples of canal habitats with a) stone bridge (junction of Llangollen and Montgomery Canal) b) open stretches of navigable water (Montgomery Canal) c) canal tunnel built in 1800's (Ellesmere Tunnel, Llangollen Canal) d) bridge built in 1990's to accommodate A663 over Rochdale Canal in Oldham, England e) bridge over Montgomery Canal f) dry section of canal (Montgomery Canal near Oswestry, England) g) one end of isolated canal (Manchester, Bolton and Bury Canal) near Bolton, England h) pollution in Newport Canal (www.shropshirestar.com).

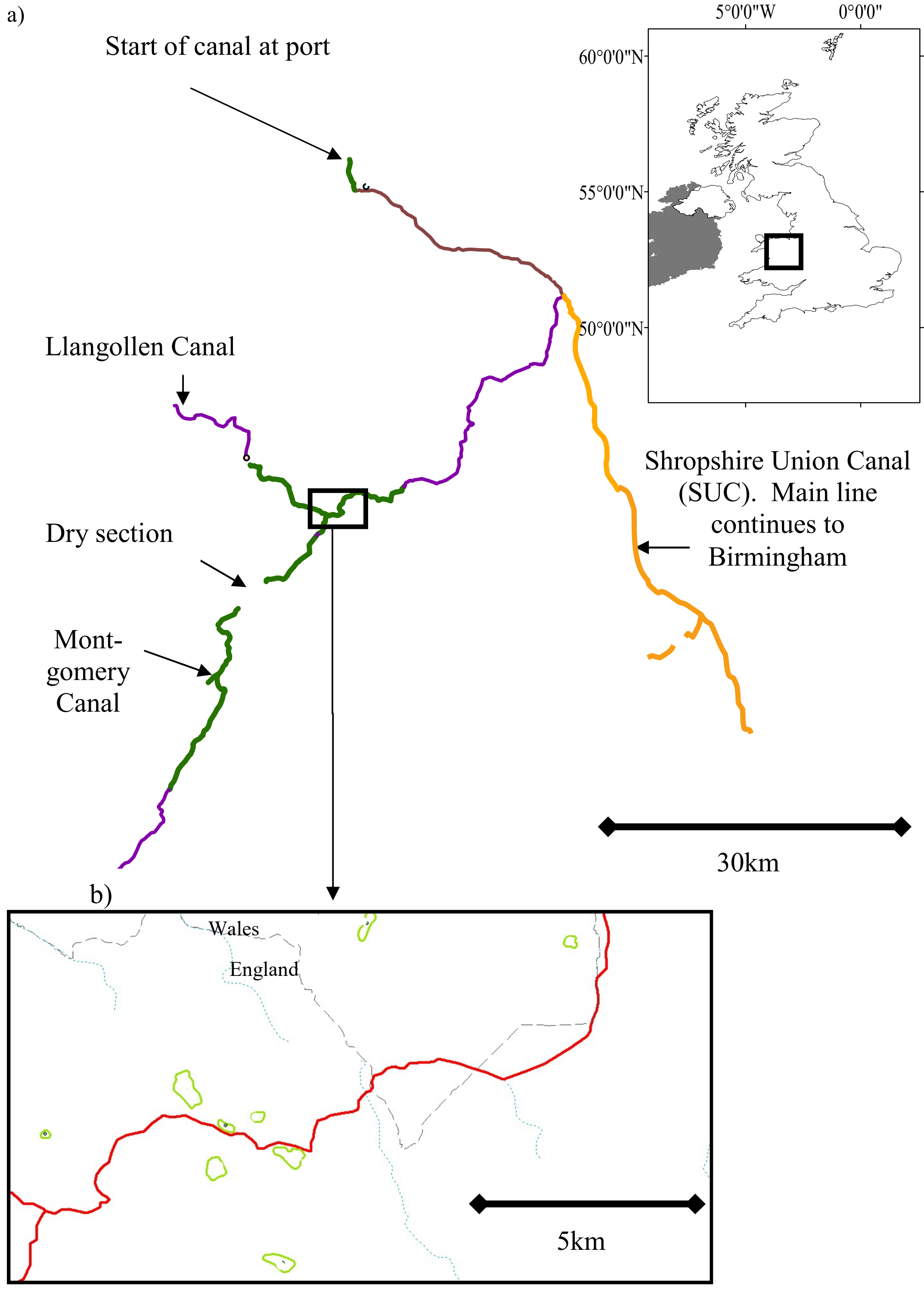


Fig. 3. a) Historic development of the Shropshire Union Canal with inset map to indicate location in the UK: canals built and opened up to 1780 (red), canals built and opened from 1781 to 1800 (green), canals built and opened from 1801 to 1820 (purple) and canals opened after 1820 (yellow).b) Enlargement of section the Shropshire Union Canal (red) with other types of freshwater habitats: lotic (blue), lentic (green).

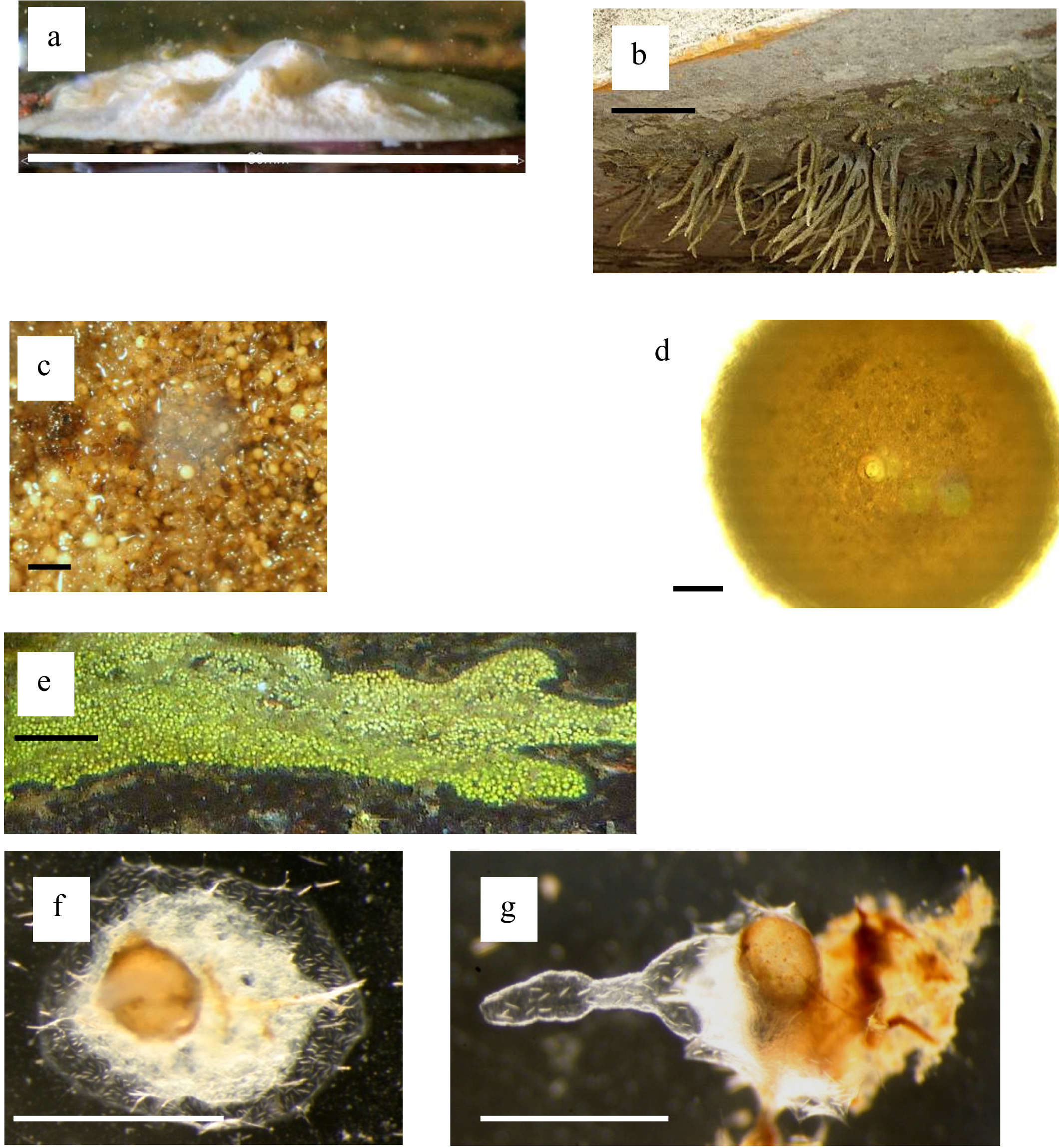


Fig. 4. Freshwater sponge colonies a) view over sponge growing on canal substrate (scale bar=0.08 m) b) *S. lacustris* colonies growing as "fingers" on underneath of canal boat (scale bar=0.1m) c) brown and white gemmules in degenerating colony of *E. mülleri* (scale bar=0.01 m) d) closer view of *E. mülleri* gemmule with foramen in centre (scale bar=0.5 mm) e) gemmules as small white "dots" in colony of *S. lacustris* (scale bar=0.01 m) f) hatched gemmule with developing sponge (scale bar=0.01 m) g) early sponge growth showing exhalent "chimney" with needle-like megascleres (scale bar=0.01 m).

Supporting Information 1.1.

Box S1. Detectability and Rarefaction

The sampling effort to determine all species at a site was considered as follows (after Williams *et al*., 2001; Krebs, 1989):

C_i = number of species counted at site i, N_i = true number of species at site i, p_i = associated detection probability

$$Expected(C_i) = p_i N_i$$

With an estimation of detection probability as

$$p_i = \frac{C_i}{N_i}$$

Following replication of sampling, the number of undetected species decreased with time. The resulting change in the number of detected new species provided information about the number of species yet to be detected. The assumption was that each species had an equal probability of detection in all samples. Thus estimate species detection probability with: n_1 = number of species in sample at time 1, n_2 = number of species in sample at time 2, m = total number of species detected in both samples, p_1 = detection probability associated with time 1as fraction of species in sample 2 that were also in sample 1, was

$$p_1 = \frac{m}{n_2}$$

These data collected were used to produce species accumulation rarefaction curves, using

$$Expected(S_n) = \sum_{i=1}^{s} \left[1 - \frac{\binom{N - N_i}{n}}{\binom{N}{n}} \right]$$

Where $Expected(S_n)$= expected number of species in a random sample of n individuals, S = total number of individuals in the entire collection, N_i = number of individuals in species i, N = total number of individuals in collection, n = value of sample size (sampling effort) ($n \leq N$), $\binom{N}{n}$ = number of combinations of n individuals that can be chosen from a set of N individuals.

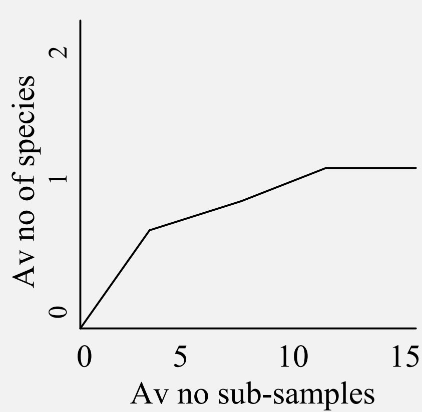
Method

To determine species richness at a site (or sub-site, see Chapter3), the sampling effort was first was assessed. Although not considered in previous sponge

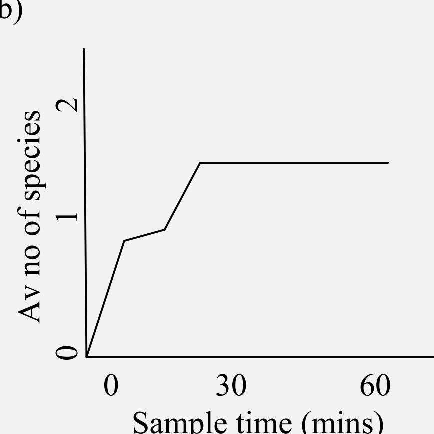
surveys (e.g. Økland and Økland, 1996, Gugel, 1999), variable detectability of sponge colonies can result in sampling errors and biased estimates of the true number of species. A rarefaction curve was obtained by systematically increasing the sample size (*n*), then plotting the sample size on x-axis against number of the sample size (*n*), then plotting the sample size on x-axis against number of new species on y-axis. At a certain point the gradient of the regression curve decreased showing the minimum sample size needed to effectively sample the population (Krebs, 1989). The total species richness is the asymptote of this curve and represents the minimum sample size needed to adequately sample the population.

Results and Discussion

a)



b)



a) Rarefaction curve with number of freshwater sponge species as sample size increases, and b) number of species accumulated by sampling effort. The sampling effort needed to locate all species present within a site was between 3 and 10 samples (typically 6), which could be collected in about 30 minutes. All data were from subsamples taken at environmentally suitable canal sites (see Chapter 2). This method supports a CPUE (catch per unit effort) technique used by Dröscher and Waringer (2007).

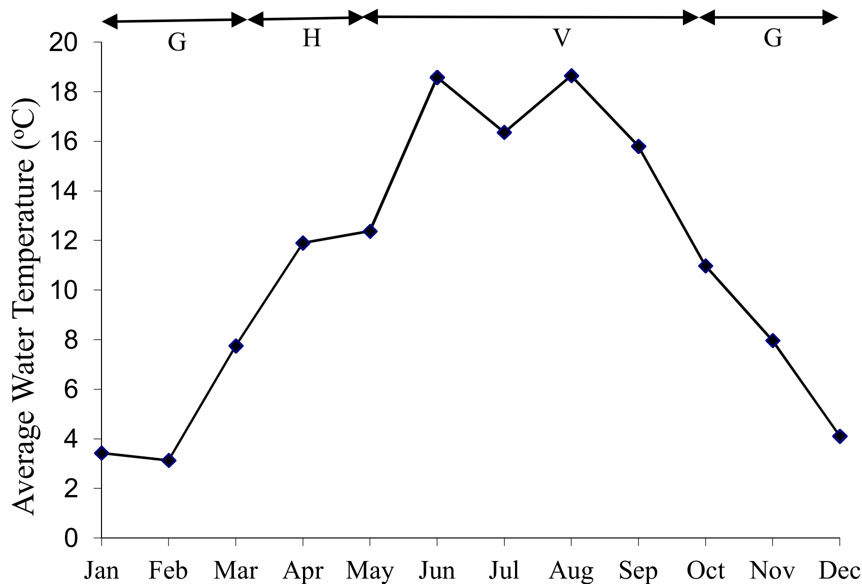


Fig. S2. Seasonal variation in average water temperature (°C) of Shropshire Union Canal during one year of study (2011) with sponge life cycle stages (G = gemmule formation and overwintering, H =gemmule hatching, V = vegetative growth and colony persistence).

Chapter 2. Identification, characterisation and distribution of freshwater sponge species (Porifera, Spongillidae) in the UK, with first documentation of *Trochospongilla horrida*.

Summary

Many aspects of freshwater biodiversity remain unknown, including species richness and current distributions of UK freshwater sponges. Although seemingly common in many habitats, freshwater sponges have been understudied in the UK. A possible reason for the scarcity of data is the difficulty in species identification. A rigorous UK taxonomic survey for freshwater sponges identified species: *Spongilla lacustris*, *Ephydatia mülleri*, *E. fluviatilis*, *Eunapius fragilis, and Racekiela ryderii*. Additionally, the freshwater sponge *Trochospongilla horrida* was reported from the UK for the first time. Both spicule characteristics and molecular data from 28S rDNA were used for identification. A taxonomic key to the freshwater sponges of the UK was constructed. Details of taxonomy, morphology, and distribution of each species were assessed. Water parameters linked with sponge presence included salinity and the occurrence of *S. lacustris*. With knowledge of the current distribution of native invertebrates, such as sponges, it is possible to identify changes in community composition over time.

Introduction

Assessing biodiversity provides a foundation for ecosystem studies (Hebert *et al*., 2003), allowing a formal assessment of how rich areas are in supporting life and how areas compare with one another. Although understanding the positive influence of biodiversity on ecosystem functioning is becoming more established (Flynn *et al*., 2011; Hooper *et al*., 2005), the extent of biodiversity in most freshwater ecosystems remains to be rigorously explored, particularly among invertebrate species (Dudgeon *et al*., 2006). This study increases our knowledge of one common, but poorly assessed (Reiswig *et al*., 2009) freshwater invertebrate group, the sponges (Porifera, Spongillidae).

Freshwater sponges are colonial, benthic invertebrates that adhere to a variety of submerged substrates (Bergquist, 1978) and play important roles in ecosystem

processes (Reiswig *et al*., 2009), including feeding selectively on a range of particles (see Reiswig *et al*., 2009). Spongillidae are widespread and often common in lakes, and rivers (Penney and Racek, 1968; Reiswig *et al*., 2009; Lim and Tan, 2013; see Chapter 1). However, there has been a lack of species documentation in UK anthropogenic habitats. The formation of canals built using solid materials (Supporting Information 2.1, Table S1) similar to those found in natural habitats, theoretically provided an expansion of microhabitats for species colonisation (Freeland *et al.* 2000). Thus, in the present study, canals, rivers, and lakes were surveyed for species presence to determine distribution patterns.

In general and especially in the UK, the freshwater sponge fauna is still poorly studied, with fragmentary records (e.g. Parfitt, 1868; Carter, 1868; Annandale, 1908; Mellanby, 1953; Clegg, 1965; Waterston, 1981; Pronzato and Manconi, 2001). Being isolated from the rest of Europe (Preece, 1995), and with extensive anthropomorphic freshwater habitat (> 3000 km, Hadfield (1959)), the UK offers a unique location to assess sponge species distribution. Within the UK there are five reported sponge species (Demospongiae, Heteroscleromorpha, Spongillidae): *Spongilla lacustris* (Linnaeus, 1759) originally known as "pond sponge"; *Ephydatia fluviatilis* (Linnaeus, 1759) also known as "river sponge" (Mellanby, 1953); *Ephydatia mülleri* (Lieberkühn, 1855); *Eunapius fragilis* (Leidy, 1851); and *Racekiela ryderii* (Potts, 1882) (= *Anheteromeyenia ryderi*) (Pronzato and Manconi, 2001). However, only three of these (*S. lacustris*, *E. mülleri*, and *E. fluviatilis*) are reported as contemporary UK species in World Porifera Database (www.marinespecies.org/porifera) (van Soest *et al*., 2014, accessed 01.01.2014). The first goal of this study was to confirm the species' list for freshwater sponges in the UK, and in doing so a sixth freshwater sponge was found: *Trochospongilla horrida* (Weltner, 1893).

A possible reason for the poor recognition of sponge species was a lack of clear morphological features that would enable ready recognition during field surveys. Difficulties in species' identification and taxonomic confusion (i.e. unrecognised or cryptic species) are two factors that result in incorrect records and an under-estimation of biodiversity (Bell and Barnes, 2001, Klautau *et al*., 1999;

Bickford *et al*., 2007). Many records of freshwater sponges, in the UK and elsewhere, lack useful taxonomic information hindering accurate species recognition (Ricciardi and Reiswig, 1993). A second goal of this study was thus to provide unambiguous characters that would allow others to recognise freshwater sponge diversity in the UK. First, sponges were identified using their morphological features including spicule skeleton characteristics. These were used to provide an identification guide for the first time in the UK, which would be useful for further research. Second, these morphological methods were supported by a molecular approach, based on sequencing a section of 28S rDNA including the variable D3 region (Alvarez *et al*., 2000; Lopp *et al*., 2007).

Once means to identify the sponges were established, a systematic study of the distribution of UK sponges was undertaken. Analysis of survey data, together with data from other sources, enabled identification of species whose apparent absence from UK habitats was due to a limited biogeographic range as opposed to a lack of sampling, or suitable habitat. The third goal was then to test the hypothesis that if the reported sponge species were found, they would co-exist at all environmentally suitable sites. The theoretical outcome would be widespread species' distribution across the UK. However, if sponges exhibit more patchy distributions, then it raises the possibility that their distributions are affected by environmental tolerances or other colonisation barriers. A further aim was, therefore, to obtain quantitative information on the water parameter correlations of sponge species.

Several studies elsewhere have investigated the presence of sponges with different water parameters, as summarised by Harrison (1974), with the most extensive data set collected in Wisconsin, USA (Jewell, 1935, 1939). *Ephydatia fluviatilis* and *Eu. fragilis* are suggested to be eurytopic, whereas *E. mülleri*, and *R. ryderii* are regarded as stenotopic (Harrison, 1974). The present study recorded similar water parameters to previous studies, i.e. temperature, conductivity, pH, total dissolved solids (TDS), (Supporting Information 2.1, Table S2), but these were extended this set by including other factors that may also affect sponge distribution: oxygen content, oxidation reduction potential, and salinity (Ricciardi and Reiswig,

1993; Williams and Fulthorpe, 2003). As salinity values have a direct effect on conductivity and TDS (Radojevic and Bashkin, 2006) this may impact on the distribution of stenotopic species. Altogether, this investigation overcomes the problems associated with a fragmented collection of reports, establishes the extent of UK sponge biodiversity, provides an indication of factors governing distribution, and establishes a baseline from which to extend the assessment of this widely spread but poorly examined component of aquatic ecosystems.

Methods

Sponge material – location and data sources

Three sources of data were used. First, a canal survey, following preliminary data collection (see below); between 2008 and 2012, surveys for freshwater sponges were performed underneath canal bridges along 16 canals at 100 stations (Supporting Information 2.1, Table S3). All stations were located along the water line and under a bridge; three stations were equally spaced along a 5 m stretch. At each station, substrate type was categorised (Supporting Information 2.1, Table S1) and examined for presence of sponges; sponge colony characteristics were recorded, and three sub-sample specimens, bearing gemmules if possible, were collected from each colony to a water depth of 40 cm. When an apparent species differed in colony growth form, the different sponge morphs were collected. Sponge samples were stored in 100% ethanol at 5 °C. Each sites was visited between 2 and 10 times, during which time the following water parameters were measured with a handheld meter (YSI 556 multiprobe system, YSI Incorporated, Ohio, USA): temperature, pH, salinity, dissolved oxygen, conductivity, total dissolved solids (TDS), and oxidation/reduction potential (ORP) (Supporting Information 2.1, Table S2). Second, a UK survey was conducted with additional sponge samples obtained from 70 locations in freshwater habitats (lakes, rivers, and 8 other canals) between 2010 and 2013, but generally without collecting water parameter data. Sponges were stored as above. See Supporting Information 2.1, Table S4 for details of habitat. Third, samples (n=50) from across the UK were obtained from the Natural History Museum, London (Supporting Information 2.1, Table S5). For all survey samples, the source location was recorded to determine their geographical distribution (Fig. 1).

Taxonomy - Microscopy

All species were identified by light microscopy. First a 1 cm^3 piece of each sub-sample was boiled in 100% nitric acid to separate siliceous spicules from tissue. After three washes in water and re-suspension in 100% ethyl alcohol, spicules were transferred to slides. Spicules were examined at 200 x magnification; images were captured with a digital camera and analysed with an image analysis system. Up to three types of siliceous spicules were analyzed for sponge identification: megascleres, which make up the framework of a sponge skeleton; microscleres, spicules with a smaller size range; and gemmuloscleres, which surround the gemmules. The minimum, maximum and mean (±SE) length and width of the spicules were determined from >25 spicules of each type from several specimens (following Volkmer-Ribeiro and de Souza Machado, 2007) except for *T. horrida* where 12 - 24 were measured. Measurements were compared with Pronzato and Manconi (2001). The following morphological characters were used as diagnostic traits: colony dimensions, external shape, colour, macro and micro morphology of skeletal mega- and microscleres, gemmule arrangement, and gemmulosclere morphology. These traits formed the basis for an identification guide (Fig. 2).

Taxonomy - Sequencing

The D3 domain of the 28S rDNA together with ~150 bp of the 3′ core sequence (303 bp) was amplified using the primers of Lopp *et al*. (2007). Total genomic DNA was extracted from sponge samples using a DNA extraction kit (DNeasy, Qiagen Inc, Hilden, Germany) following the manufacturer's instructions, except that after incubation in lysis buffer the tubes were briefly centrifuged to remove spicules. DNA concentration was quantified using a QuantiT dsDNA assay kit and a Qubit fluorometer (Invitrogen, Carlsbad, CA, USA). PCRs were set up in a 20 µl final reaction volume that contained ~ 10 ng genomic DNA containing the following: 10 µl 2X GoTaq Green (Promega, Southampton, UK), 0.4 pmole of each primer (Eurofins MWG Operon, Germany), 2.0 µl 10X BSA buffer (New England Biolabs Inc., Hitchen, UK). Thermal cycling conditions were: 94 °C for 4 min, followed by 35 cycles at 94 °C for 45 s, 45 °C for 30 s, 72 °C for 45 s, and a final 72 °C for 8 min. PCR products were then purified with 0.15 µl shrimp alkaline phosphatise (1000U ml^{-1}) (USB, UK) and 0.03 µl Exonuclease I (20000Uml^{-1}) (New

England BioLabs, UK) following manufacturer's protocol. Sequencing was performed using BigDye v.3.1 chemistry (Applied Biosystems, Life Technologies, Paisley, UK), with ~ 10 ng of PCR products and 1.6 pmol of primer (forward or reverse) in each reaction. Sequencing products were cleaned by ethanol precipitation, and then separated by capillary electrophoresis on an ABI3100xl. Any potentially contaminated samples (e.g. with bacterial DNA sequence) were re-sequenced. Forward and reverse sequences were checked by eye in Geneious v.6.1.2 (www.geneious.com, Kearse *et al*., 2012) to produce a consensus. Individual sequences were aligned using MEGA v.5 (Tamura *et al*., 2011), and the consensus sequences for each species were then aligned using CLUSTALW v. 2.0.12 (Thompson *et al*., 1994). Sequences were then manually checked and trimmed; alignments were then subsequently revised by eye in an effort to maximize positional homology. Pairwise genetic distances between species were calculated using Kimura's two-parameter (K2P) model (Kimura, 1980) using MEGA v.5.

Statistical analysis

For all data, two analyses were used with SPSS v. 22 (IBM Corp, 2013) to assess similarities in presence of species at sites: 1) Cochran's Q test (for dichotomous variables; Zar, 2010) was used to test for heterogeneous distributions followed by a post-hoc Bonferroni test (Zar, 2010), contrasting co-existing species; 2) the degree of sponge species co-existence among sites was assessed by cluster analysis on the overall Jaccard coefficient J, where $J = (a/[a + b + c])$; a is the number of species present at two sub-sites, and b and c are the total number of species occurring at each sub-site but not the other (see Clifford and Stephenson, 1975; Plafkin *et al*., 1989). Additionally, to identify significant water parameters for sponge presence, data from the Canal Survey were analysed with SPSS v. 22 using t tests for independent samples together with Levene's test examining the assumption of equal variances (Zar, 2010).

Results

Occurrence

Six freshwater sponge species were found: *Spongilla lacustris*, *Ephydatia mülleri*, *E. fluviatilis*, *Eunapius fragilis, Racekiela ryderii*, and *Trochospongilla*

horrida. Spicule morphology was unambiguous in species identification. This is the first documentation of *T. horrida* in the UK. Sponges occurred alone and with other freshwater sponge species, in full light, in shade, or in dark habitats. Adult sponges and/or gemmules occurred on permanent, established, submerged, substrate; most commonly they occurred on brick, rock, or concrete substrate (Fig. 3). Few adult sponges grew on macrophytes, and no gemmules occurred on permanent plant material. Sponge larvae were rarely observed when observing colonies.

Taxonomy

Species identification was based on colony characteristics, spicule morphology (Table 1, Fig. 4; Supporting Information 2.1, Table S6), and genetic divergence (Fig. 5). Although problems occurred when sequencing *T. horrida*, consensus rDNA 28S sequence data (from the D3 domain) were obtained only for *S. lacustris, E. fluviatilis, E. mülleri, Eu. fragilis* and *R. ryderii* (Fig. 5). Sequences differed at 12 sites with pairwise sequence divergence across all sequences was low, ranging between 0.000 and 0.017 (mean distance 0.007). Sequence data for *T. horrida* were not obtained, largely due to a combination of small colony size (i.e. limited DNA sample) and the sample itself was contaminated by bacteria; a single colony was recorded in June, mixed with a colony of *E. mülleri*, at a canal site in the south of England (Countess Wear bridge, Exeter Canal) (Fig. 6e).

Species characteristics

Spongilla lacustris (Linnaeus, 1759)

Colony characteristics: Encrusting colonies (l to 2.5 cm thick) occurred with irregular, rounded edges. Colonies were often green with a ribbed or astrorhiza surface (Fig. 7a), disintegrating in October to November, leaving white or brown gemmules in former basal parts. At some sites, colonies were 1 m long (June to August) and occasionally formed arborescent finger-like projections (10 x 1 cm) (Fig. 7b). It occurred in lake and canal habitats, occasionally occurring (<33% of sites) with zebra mussels at canal sites.

Spicule characteristics: Megascleres were straight or slightly curved, smooth oxeas (Fig. 4). *Spongilla lacustris* is the only UK species with microscleres. These were slightly curved oxeas, with micro-spines. Gemmuloscleres were slightly to strongly

curved with spined oxeas or strongyles, (Fig. 4). Microscleres and gemmuloscleres were shorter than those described by Pronzato and Manconi (2001) (Table 1).
Geographical distribution: Widely distributed throughout UK (Fig. 6a).

Ephydatia mülleri (Lieberkühn, 1855)
Colony characteristics: Firm, thickly encrusting (2-4 cm thick) colonies occurred, with irregular, rounded edges (Fig. 7d). Colonies were yellow, brown, or pale grey and often hispid. Most colonies were located at undisturbed sites, peaking during late summer (July to August). Colonies partially disintegrated in winter, leaving gemmules covering former basal parts.
Spicule characteristics: Megascleres were slightly curved oxeas, with micro-spines (Fig. 4); the tips were often smooth. Gemmuloscleres were rotules with a shorter shaft than those described (20–12 μm) by Pronzato and Manconi (2001) (Table 1). Rotules were irregularly and deeply incised into <10 rays.
Geographical distribution: England, Wales, and N. Ireland (Fig. 6b) and less widely distributed than *S. lacustris*.

Ephydatia fluviatilis (Linnaeus, 1759)
Colony characteristics: Encrusting colonies formed an irregular outline and rounded edges. Large green, lobate colonies (>0.3 m long) were occasionally found at open canal sites. In rivers, smaller ecru colonies (<10 cm diameter) were conspicuous against dark substrate (usually rocks) in shaded areas (Fig. 7c). Small colonies were located on underneath surfaces of stones at lake sites. Some colonies were devoid of gemmules.
Spicule characteristics: Megascleres were typically slightly curved, smooth oxea of a similar size to those described by Pronzato and Manconi (2001) (Table 1). Gemmuloscleres were birotules with occasionally spined shafts (23-33 μm) and >10 rays (Fig. 4).
Geographical distribution: Throughout England, also Scotland, and Wales (Fig. 6c).

Eunapius fragilis (Leidy, 1851)
Colony characteristics: Colonies were low crusts (~ l-2 cm thick), with an irregular, rounded outline. Colony surface was often ribbed, with noticeable oscula.

Numerous, small (rarely >20 cm), ecru colonies were recorded at some sites, and these disintegrated in September to October. Gemmules occurred in a pavement layer in a characteristic pattern for this species i.e. in "carpets", or in individual clusters (2-4) (Pronzato and Manconi, 2001). Foramina occurred as short tubes, always directed upward from the pavement layer or outward from the cluster (Fig. 7e).

Spicule characteristics: Megascleres varied from straight, to slightly curved smooth oxeas. Gemmuloscleres were slightly curved or straight spiny oxeas (Fig. 4), shorter than those described by Pronzato and Manconi (2001) (Table 1).

Geographical distribution: England and Wales (Fig. 6d), with a similar distribution to *E. mülleri*.

R. ryderii (Potts, 1882)

Colony characteristics: Thin hemispherical encrustations (<1 cm thick) occurred, with rounded edges and a hard, brittle consistency. It occurred in lakes, growing on the top surface of rocks, in large numbers, peaking during July to August. Colonies contained a small number of gemmules. In several samples (UK Survey data), it was associated with *S. lacustris*.

Spicule characteristics: Megascleres were variable among specimens; exhibiting differences in robustness, length ranging from 350 to 160 µm, and width from 4 to 14 µm (see Chapter 6). In general, megascleres were amphioxea covered with short pro-curved spines, except near the tips (Fig. 4), although there were variations in spination. Two distinct classes of gemmuloscleres occur (Pronzato and Manconi, 2001), and these were present in small numbers but were shorter than those described by Pronzato and Manconi (2001) (Table 1).

Geographical distribution: Sites in England, Northern Ireland, Scotland and Wales (Fig. 6e).

Trochospongilla horrida (Weltner, 1893)

Colony characteristics: Colonies were thin (<5 mm), flat, light yellow encrustations.

Spicule characteristics: Megascleres were amphioxea, densely covered with spines (Fig. 4). Megasclere tips were sparsely spined, and a similar size range to those described by Pronzato and Manconi (2001) (Table 1). Gemmoscleres were

birotulates with short, smooth shafts. Rotules were disk-like, with smooth, entire margins, nearly equal in diameter. The overall length of gemmulosclere was never greater than diameter of smaller rotules.
Geographical distribution: Rare in the UK; recorded at one canal site in southern England (Countess Wear bridge, Exeter Canal) (Fig. 6e).

Overall sponge distribution

Species distributions were heterogeneous (Fig. 6) with significant differences in species' presence at sites ($X^2_{(5)} = 115.6$, p <0.01; Table 2). The most densely populated regions of distribution are the ones covered by Canal Survey data. These data enable differentiation between species with wide biogeographic ranges (*S. lacustris*, *E. fluviatilis*), those with more patchy distribution (*E. mülleri*, *Eu. fragilis*), and rare species (*R. ryderii*, *T. horrida*). Both *R. ryderii* and *T. horrida* occurred at significantly fewer sites than the other species (p <0.01). There was a significant difference between the distribution of *S. lacustris* with *E. mülleri* and/or *Eu. fragilis* at sites (p <0.01). *Spongilla lacustris* only occurred in lakes and canals whereas *E. mülleri* and *Eu. fragilis* occurred mostly in lakes and canals, and occasionally (<10% of sites examined) in rivers (Fig. 8). Over all sites, the most frequently coexisting pair of species was *Eu. fragilis–E. mülleri*, (J=0.24), although *Eu. fragilis* also occurred with *S. lacustris* and *E. fluviatilis* (both J=0.17). Over all species, *E. fluviatilis* most commonly occurred in rivers (Fig. 8). *Racekiela ryderii* occurred at nearly 6% of sites (lakes), without other species (J=0.02–0.04) (Fig. 8). As *T. horrida* occurred at one site, this species has the lowest overall values of J (J<0.03; Table 3).

Although all sites in the canal survey were constructed from a limited number of substrates (Supporting Information 2.1, Table S1), 51% of sites did not contain sponges. Colony occurrence for *S. lacustris*, *E. mülleri*, *E. fluviatilis*, and *Eu. fragilis* was highest during the summer when water temperature was significantly higher ($\bar{x}$=12.10°C, SE±0.33, p >0.01) than in winter, and dissolved oxygen levels were significantly lower ($\bar{x}$=10.56 mgl^{-1}, SE±0.43, p >0.01) than in winter. At canal survey sites where colonies were present, temperature ranges were similar for *S. lacustris*, *E. fluviatilis*, and *Eu. fragilis* (from 7.2°C to 18.1°C) (Supporting

Information 2.1, Table S7), with highest colony occurrence linked to higher water temperatures (Table 4). When water temperatures were lower (in winter) adult colony structure degenerated and thus sponges did not occur or only gemmules were present. Canal survey sites with sponges also had significantly (p >0.05) lower salinity ($\bar{x}$=0.15ppt, SE±0.02) than those without colonies. Despite significantly differently distributions, *S. lacustris* and *E. mülleri* were both recorded from sites with lower average conductivity, TDS (Supporting Information 2.1, Table S7) and significantly lower salinities (Table 4). *Spongilla lacustris* occurred most often at sites when ORP levels were significantly more positive ($\bar{x}$ = 4.71 mV, SE±3.47, p >0 .05) and water conductivity was significantly lower ($\bar{x}$ = 180.48 µScm^{-1}, SE±24.04, p >0 .01) (Table 4). Both *E. fluviatilis* and *Eu. fragilis* were recorded at sites with more negative average ORP levels than *S. lacustris* and *E. mülleri* (Supporting Information 2.1, Table S7). Also, average oxygen concentrations were lower at sites with *E. fluviatilis* than for *S. lacustris*, *E. mülleri* and *Eu. fragilis* (Supporting Information 2.1, Table S7). Although only found at one site, *T. horrida* was associated with low conductivity (<500 µScm^{-1}), and also with slightly alkaline pH. *Racekiela ryderii* occurred with salinity, conductivity, and TDS values that approached zero, and with high positive ORP (>100 mV) values.

Discussion

Sponges are an important part of UK freshwater fauna: they increase invertebrate biodiversity (Manconi and Pronzato, 2008), and affect habitat structure and environmental quality through association with other invertebrates and filter-feeding capacity (Reiswig *et al*., 2009). Present UK distribution records of freshwater sponges are based on serendipitous finds, with no systematic examination of their distribution or potential underlying drivers such as habitat tolerances. This study now provides evidence for six freshwater sponge species in the UK, demonstrating that freshwater sponges are more diverse in the UK than previous records have indicated. Freshwater sponge biodiversity is still lower than that of many other freshwater invertebrate taxa, e.g. 19 species of bryozoans (Wood and Okamura, 2005) and 12 species of flatworm (Mellanby, 1953). With fewer sponge species, the possibility arises that species are generalists and are located in all habitats (see Manconi and Pronzato, 2007). This survey investigated the possibility

that the five reported sponge species would occur and would be homogeneously distributed; and then (given that they were not homogenous) assessed if species occurrence depended upon water chemistry. These findings provide the foundation for future evaluation of species distribution patterns.

The second purpose of this survey was to develop a morphology-based key to UK species; one that should also apply more broadly to Europe, as these species are wide spread (Pronzato & Manconi, 2001). This key pays particular note of megasclere characteristics, as these are always present in the adult sponges, and it is designed to provide relatively fast identification, especially for those with lower levels of expertise. However, there are difficulties that occur with using just megasclere similarities, e.g. there are few differences between *E. fluviatilis* and *Eu. fragilis*. This reinforces the need for molecular data to be applied to sponge species' identification.

Increasingly, molecular data are able to shed new light on taxonomy (see Hebert *et al*., 2002; Folmer *et al*., 1994) using sequence data from several gene partitions of mitochondrial and nuclear DNA. In the present study, the D3 expansion segment of 28S rDNA, (see Alvarez *et al*., 2000; Lopp *et al*., 2007) is used to overcome limitations of morphological similar features (Hooper and van Soest, 2002) and conclusively identify species. Although problems occurred when sequencing *T. horrida* (as outlined in the Results), consensus rDNA 28S sequences were obtained for five species. Distance analysis of consensus rDNA 28S sequences used the Kimura-two-parameter (K2P) model as it is suggested to be the best metric when distances are low (Hebert *et al*., 2002) as in this study. Low K2P values for five sponges can be the result of either slow mitochondrial evolution or differences in speciation rates (Zhang *et al*., 2011) and suggest a common origin for these species (Hebert *et al*., 2002). Values of K2P are low compared with other invertebrate studies using this marker (e.g. Zou *et al*., 2012, Zhang *et al*., 2011) and with other sponge studies (Itskovich *et al*., 2013). This analysis together with a wider molecular analysis of Demospongiae from across Europe using other genetic markers may provide insights into the radiation of Demospongiae (Manconi and Pronzato, 2007).

Sponge species biogeography

Freshwater sponges are not ubiquitous in UK habitats, with species considered as common, patchy or rare. Of the six UK species, *S. lacustris* was the most widespread sponge in the UK (34.1% of sites), consistent with other data (Ricciardi and Reiswig, 1993; Økland and Økland, 1996; De Santo and Fell, 1996) where it was often recorded in lakes in keeping with its specific name 'lacustris'. However, here *S. lacustris* was also common in canals at shaded and open sites. Some *S. lacustris* colonies were green from chlorophyll contained within populations of algal symbionts (Reiswig *et al*., 2009), a feature especially noticeable at open sites. This endosymbiosis is an apparently successful trophic strategy, playing a major role in colony persistence of some species (Reiswig *et al*., 2009). *Spongilla lacustris* occurred in habitats with low conductivity, TDS, and salinity levels, in general agreement with data from other areas (Harrison, 1974). This suggests that ions contributing to salinity (and indirectly to conductivity and TDS values) (Radojevic and Bashkin, 2006) form limiting conditions for this species, with no occurrence in brackish habitats or those with influences by salt influx. The other common species, *E. fluviatilis*, was frequently present (22%) at sites, and is the most common species worldwide (Lopp *et al*., 2007). The specific name 'fluviatilis' indicates occurrence in rivers, but while *E. fluviatilis* was the species most often recorded from rivers, this species was located in canals and lakes also. Some colonies did not exhibit gemmules possibly affecting persistence at a site, as, in common with other surveys (Stephens, 1920; Gugel, 1999; Lucey and Cocchiglia, 2014), some years *E. fluviatilis* was absent at habitats where it had previously been abundant.

The rarer species, *Eu. fragilis* and *E. mülleri*, exhibited a patchy distribution within UK freshwater habitats. An apparently patchy distribution may occur because colony characteristics result in a species being overlooked (Dröscher and Waringer, 2007); for example, colonies of *Eu. fragilis* were smaller, were not easily distinguished from the substrate, and started to disintegrate earlier in the year than other species in the UK and in Europe (Tendal, 1967; Gugel, 2001; Dröscher and Waringer, 2007). A patchy distribution may also occur if species persistence is influenced by habitat parameters not measured in the present study. For example, a

link with increased *E. mülleri* occurrence and a low level of habitat disturbance has been reported (S. Leys pers comm). However, the detailed assessment of the optimum level of sampling required for an unbiased survey in the present study ensured a wide variety of habitats were surveyed, and patchy species were not overlooked. In fact, *E. mülleri* exhibits a patchy distribution pattern throughout Europe (Stephens, 1920; Tendal, 1967; Waterston, 1981; Manconi and Pronzato, 2008). In addition, an unbiased survey ensured that an accurate record of the high levels of co-existence between *Eu. fragilis* and *E. mülleri* (also reported by Ricciardi and Reswig, 1993).

Although *T. horrida* is reported as rare (Richelle-Maurer *et al.*, 1994), its presence in the UK could have been predicted from examining rigorous freshwater sponge surveys elsewhere in Europe (Dröscher and Waringer, 2007; Gugel, 1999). Despite a cosmopolitan distribution (Table 5), this first record of *T. horrida* in the UK could represent an expansion of its range from central Europe to a southern UK habitat. The record of *T. horrida* at a coastal canal site corresponds to a tolerance for brackish water (Poirrier, 1969). One main characteristic of *T. horrida* is its fast growth rate (Gugel, 1999), growing over other species. This could result in spicule mixes during morphological investigations. *Trochospongilla horrida* has spined, oxea megascleres and *E. mülleri* has micro-spined, oxea megascleres, both with overlapping dimensions. Thus, without characteristic gemmuloscleres, possible taxonomic uncertainties may arise. The absence of a published 28S rDNA *T. horrida* sequence adds to this uncertainty, thus further *T. horrida* specimens are required to overcome this. Sub-sampling *E. mülleri* colonies from brackish sites along the south coast of the UK, and sampling in warmer months such as July, when maximum *T. horrida* is abundant in Germany (Gugel, 2000) may result in additional data.

The other rare species, *R. ryderii*, is common in the Republic of Ireland (Pronzato and Manconi, 2001; Lucey and Cocchiglia, 2014; Stephens, 1920), so perhaps it is not surprising that it can tolerate a range of water parameters in the UK. In the present study, data for *R. ryderii* represent additional information compared to the previous confirmed records from the Hebrides (Annandale, 1908; Waterston and Lyster, 1979; Waterston, 1981) (see Chapter 6). This species presence at one upland

lake (UK survey data) corresponded with high ORP associated with unpolluted water (Dodds, 2002). An upland habitat preference and a reported absence from polluted sites (Harrison, 1974; Lucey and Cocchiglia, 2014) may correspond to the absence of this species from canals.

The co-occurrence of different sponge species is common (Richelle-Maurer *et al*. 1994); the present study found *Eu. fragilis* associated with *E. fluviatilis*, a result also reported by Pronzato and Manconi (2001). This may be attributed to the cosmopolitan distributions of these species (Harrison, 1974; Pronzato and Manconi, 2001) (Table 5) and wide tolerance ranges (summarized by Harrison, 1974) within the water parameters recorded. However, the association of *S. lacustris* with *E. mülleri* and *E. fluviatilis* reported elsewhere (Pronzato and Manconi, 2001) occurred less frequently, resulting in lower values of J ($J \leq 0.15$). In fact, *S. lacustris* had a significant difference in distribution to *E. mülleri*. Some large *S. lacustris* colonies may contribute to this result as, at some sites, this species occupied all available substrate, reflecting its ability to rapidly colonise (Frost *et al*., 1982) and dominate a community (Frost, 1978). Also, unlike *E. mülleri* and *E. fluviatilis*, *S. lacustris* was not recorded from river habitats. Although other surveys have noted *S. lacustris* from river habitats, increased growth in lentic habitats was reported (Ricciardi and Reswig, 1993).

Adequate sampling and the importance of substrate

An apparent absence of freshwater sponge records caused by insufficient search is problematic in establishing bio-geographical patterns (Smith and Wilkinson, 2007). Without extensive sampling during summer, sponges may be overlooked at a site because of seasonal colony degeneration. As in other research (De Santo and Fell, 1996), a large percentage of environmentally similar sites (51%) (Canal and UK survey data) remained unoccupied. However, sponge habitat is widely distributed within the areas where colonies were not found. In the canal survey data, it is unlikely that species were overlooked, but other gaps in data from Natural History Museum may be due to sampling bias.

Despite space limitations in some locations, a large percentage of seemingly potentially suitable sites (51%) (canal survey and UK survey data) remained unoccupied. As with other freshwater invertebrates, sponges may be absent from an otherwise suitable freshwater habitat because of substrate characteristics (Minshall, 1984). Along shores of lakes and river edges, sponges usually occurred on the top surface of stones. However, during field work it became clear that sponges were more often present on shaded, vertical canal substrate than in lakes and rivers, and thus these were the most intensively researched areas. Canal substrates were particularly suitable for sponges because they allowed for gemmule settlement over a broad area over rock, brick, and concrete (Fig. 3). Broad areas of substrate rather than small particles may be an important factor for colonisation, as suggested by Gugel (1999). It is possible that species were absent from other habitats because there was no available hard substrate, or if there was, water velocity prevented gemmule settlement and colonisation. Data sources indicated there was a limited range of substrate types, and therefore there was a degree of similarity between sites. Thus, substrate alone does not explain species heterogeneities.

Water parameter barriers to sponge persistence

Together with suitable substrate, species' tolerances to water parameters, such as salinity may impact the distribution of some sponge species, e.g., *E. fluviatilis* (Belas *et al*., 1989). This UK-based study is the first intensive investigation of water parameters on sponge species distribution, and has revealed a significant effect of salinity on presence of *S. lacustris* and *E. mülleri*. Salinity generally accounts for a large proportion of the conducting ions and can mirror the level of conductivity (Mackereth *et al*., 1978; Radojevic and Bashkin, 2006), hence the significance of conductivity for *S. lacustris* presence. Also, this investigation is the first report of a link with ORP and a freshwater sponge species, specifically, *S. lacustris*. Values of ORP, together with pH, have a direct effect on sponges through control of cellular pathways and an indirect effect by affecting which bacteria (food availability) can survive (Barton and Northrup, 2011). There is a significant effect of presence of *S. lacustris* at sites with more positive ORP (Supporting Information 2.1, Table S7). Positive ORP values reflect an oxidising environment (Dodds, 2002) and this tends to positively impact benthic animals (Cai *et al*., 2011) because of high

levels of dissolved oxygen. It was surprising that only *S. lacustris* was affected and suggests this species has a different tolerance range than other UK species.

Although water temperature and dissolved oxygen were significant factors linked with sponge presence and species presence in the canal survey, it seems unlikely these parameters form a barrier to sponge presence in other habitats. Instead, temporal variation in temperature affects sponge life cycle (Harrison 1974; Poirrier 1974; Van de Vyver and Willenz 1975; Bergquist 1978). In the present study, during the summer (June to August) when water temperatures were higher, adult sponges were present; while in winter (November to February) the adult sponge body breaks down, often leaving gemmules. This temporal variation in water temperature may also correspond to dissolved oxygen levels, which were significantly lower at sites with *S. lacustris*, *E. mülleri*, and *E. fluviatilis*. Some species with symbiotic algae may be limited by seasonal light intensity changes. However, during the summer, when adult sponges are present, oxygen levels may be lower because of an increase in water temperature (Radojevic and Bashkin, 2006). Higher oxygen levels may be present in areas with water aeration (Radojevic and Bashkin, 2006) such as rivers. *Eunapius fragilis*, *E. mülleri* and *E. fluviatilis* were all represented in rivers reflecting a positive influence of water movement on colony persistence (Jewell, 1935; Richelle-Maurer *et al*., 1994; Harrison, 1974). Although sites with *Eu. fragilis* have higher average dissolved oxygen values, it was not a significant water parameter, suggesting broad oxygen tolerances.

Implications for sponge biogeography

Results from the present study increase knowledge of UK biodiversity in this group; although there are relatively few species of freshwater sponge, they are morphologically and genetically distinctive. These data provide baseline information on the distribution of sponges which is a prerequisite for future monitoring. The identification of sites with a high biodiversity may be useful in some freshwater studies e.g. to quantify the impacts of human actions (see Lévêque *et al*., 2005). However, the present study illustrates that it is important not just to value sites of high biodiversity as sites with only one species (e.g. *T. horrida*) may be useful to identify if those species are rare in the UK. As the distribution of sponges can be

considered in terms of individual species, rather than just the broad-scale patterns, biodiversity studies should ensure adequate sampling methods, and summer sampling, to allow the encounter with rare species (Lawler *et al*., 2003; Faith and Norris, 1989). All sponge species in the present study have wider distributions in the UK than previously known, but there are still apparent large areas of suitable habitat that lack some species. In other European countries, sponge species have disappeared from water bodies due to changes in water chemistry (Dröscher and Waringer, 2007), and this study has demonstrated intraspecific salinity tolerance variations. However, sponge persistence may increase with water temperature rises and by the creation of artificial hard substrates such as canal construction. Overall, the distribution of sponge species in the UK may be considered to be regulated by (1) habitat/substrate characteristics, and (2) the water parameter tolerances of the species.

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Table 1. Variation in spicule dimensions indicating mean length and width from the present study (n>25) and compared with those from Pronzato and Manconi (2001). Gemmulosclere measurements were not available (n/a) for *E. fluviatilis*, *E. mülleri* and *T. horrida*.

	Spicule type		Length (µm)		Width (µm)		Rotule diameter (µm)	
			Max	Min	Max	Min	Max	Min
Spongilla lacustris	Megascleres	This study	278	140	15	4	___	___
		Pronzato Manconi	300	90	18	2	___	___
	Microsclere	This study	91	35	7	2	___	___
		Pronzato Manconi	178	25	8	2	___	___
	Gemmulosclere	This study	89	29	9	2	___	___
		Pronzato Manconi	130	21	10	1	___	___
Ephydatia mülleri	Megascleres	This study	270	158	16	4	___	___
		Pronzato Manconi	350	200	20	9	___	___
	Gemmulosclere	This study	14	7	17	2	23	12
		Pronzato Manconi	10	5	n/a	n/a	20	12
Ephydatia fluviatilis	Megascleres	This study	419	244	16	5	___	___
		Pronzato Manconi	400	210	19	6	___	___
	Gemmulosclere	This study	33	23	6	2	25	17
		Pronzato Manconi	30	26	n/a	n/a	21	18
Eunapius fragilis	Megascleres	This study	360	175	21	2	___	___
		Pronzato Manconi	270	180	15	4	___	___
	Gemmulosclere	This study	87	54	8	3	___	___
		Pronzato Manconi	140	75	8	3	___	___
Racekiela ryderi	Megascleres	This study	352	160	14	4	___	___
		Pronzato Manconi	431	141	26	12	___	___
	Gemmulosclere	This study	23	25	5	3	21	15
		Pronzato Manconi	49	28	8	3	29	20
	Pseudo-gemmulosclere	This study	41	25	6	2	17	9
		Pronzato Manconi	92	47	10	5	23	17
Trochospongilla horrida	Megasclere	This study	230	187	10	7	___	___
		Pronzato Manconi	235	170	15	11	___	___
	Gemmulosclere	This study	20	8	5	2	17	9
		Pronzato Manconi	n/a	n/a	n/a	n/a	n/a	n/a

Table 2.Variations in percentage occurrence (%) at sites (*n*=220), indicated a significant difference in sponge species presence at sites (Cochran's Q $X^2_{(5)} = 115.6$, $p < 0.01$). Bonferroni corrected pairwise comparisons between species determined where significant differences ($p < 0.01$, $\phi = 0.003$) (=**s**) were found.

	%	*E. mülleri*	*E. fluviatilis*	*Eu. fragilis*	*R. ryderii*	*T. horrida*
S. lacustris	34.1	**s**	ns	**s**	**s**	**s**
E. mülleri	16.6		ns	ns	**s**	**s**
E. fluviatilis	22.0			ns	**s**	**s**
Eu. fragilis	18.4				**s**	**s**
R. ryderii	5.8					**s**
T. horrida	0.4					

Table 3. Jaccard coefficient (=*J*) measures of mean similarity to analyse co-existence of sponge species (*E. mülleri*, *E. fluviatilis*, *S. lacustris*, *Eu. fragilis* and *R. ryderii*) at all sites.

	E. mülleri	*E. fluviatilis*	*Eu. fragilis*	*R. ryderii*	*T. horrida*
S. lacustris	0.15	0.09	0.17	0.04	0.00
E. mülleri	1.00	0.09	0.24	0.02	0.03
E. fluviatilis		1.00	0.17	0.00	0.00
Eu. fragilis			1.00	0.00	0.00
R. ryderii				1.00	0.00

Table 4. Independent t-test analyses for presence of any sponge colony, and for presence of four sponge species, with significant abiotic water factors and significance levels (*p*). Average values of water factors with standard error (S.E.) also shown.

	Abiotic factor	t	df	sig	Mean values ± S.E.	
					Presence	Absence
Overall presence or absence of sponges	Temperature	-3.74	93.49	$p<0.01$	12.10±0.33	9.69±0.56
	Oxygen	3.45	112	$p<0.01$	10.56±0.43	13.01±0.41
	Salinity	2.61	81.91	$p<0.05$	0.15±0.02	0.26±0.04
Spongilla lacustris	Temperature	-2.55	98.8	$p<0.05$	11.97±0.41	10.42±0.45
	Oxygen	2.00	112	$p<0.05$	11.06±0.49	12.41±0.38
	Salinity	3.61	111.36	$p<0.01$	0.12±0.02	0.24±0.03
	Conductivity	-2.96	81.42	$p<0.01$	180.48±24.04	275.35±21.15
	ORP	-2.23	110	$p<0.05$	4.71±3.47	-5.91±2.71
Ephydatia mülleri	Temperature	-3.48	72.4	$p<0.01$	12.50±0.45	10.36±0.41
	Oxygen	3.33	112	$p<0.01$	10.19±0.55	12.56±0.35
	Salinity	3.03	89.25	$p<0.05$	0.12±0.02	0.23±0.03
Ephydatia fluviatilis	Temperature	-2.18	113	$p<0.05$	12.43±0.58	10.52±0.39
	Oxygen	3.50	112	$p<0.01$	9.83±0.64	12.51±0.33
Eunapius fragilis	Temperature	-2.57	113	$p<0.01$	12.35±0.49	10.36±0.42

Table 5. Results of freshwater sponge species (Family Spongillidae) identified in the present study, with reported zoogeography (Pronzato and Manconi, 2001). Although *Ephydatia mülleri* is considered a Holartic species (Pronzato and Manconi, 2001) it has also been reported from Zimbabwe (Ezcurra de Drago, 1975).

Species	UK species?	Distribution		
		Cosmopolitan	Holartic	Amphiatlantic
Ephydatia mülleri (Lieberkühn, 1856)	yes		✓	
Ephydatia fluviatilis (Linnaeus, 1759)	yes	✓		
Eunapius fragilis (Leidy, 1851)	yes	✓		
Racekiela ryderii (Potts, 1882)	yes			✓
Spongilla lacustris (Linnaeus, 1759)	yes		✓	
Trochospongilla horrida (Weltner, 1893)	no		✓	

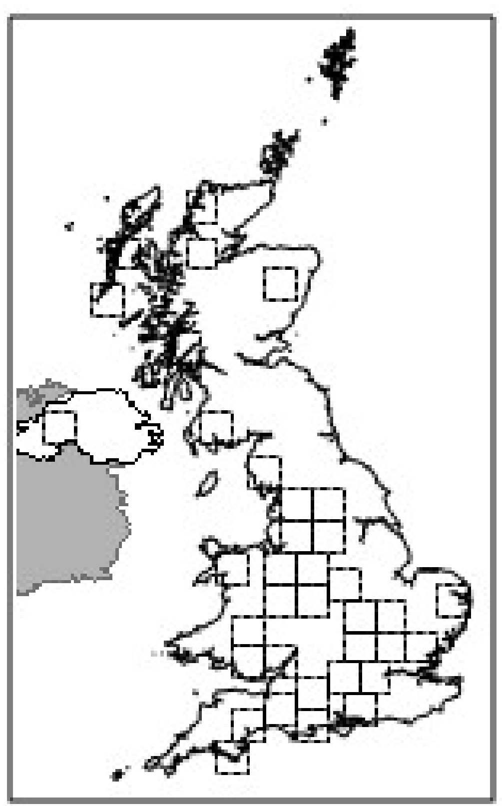

Fig. 1. Areas of UK showing surveyed (shown as squares) for the presence of freshwater sponges (N = 220), and includes areas where sponges were found by other researchers.

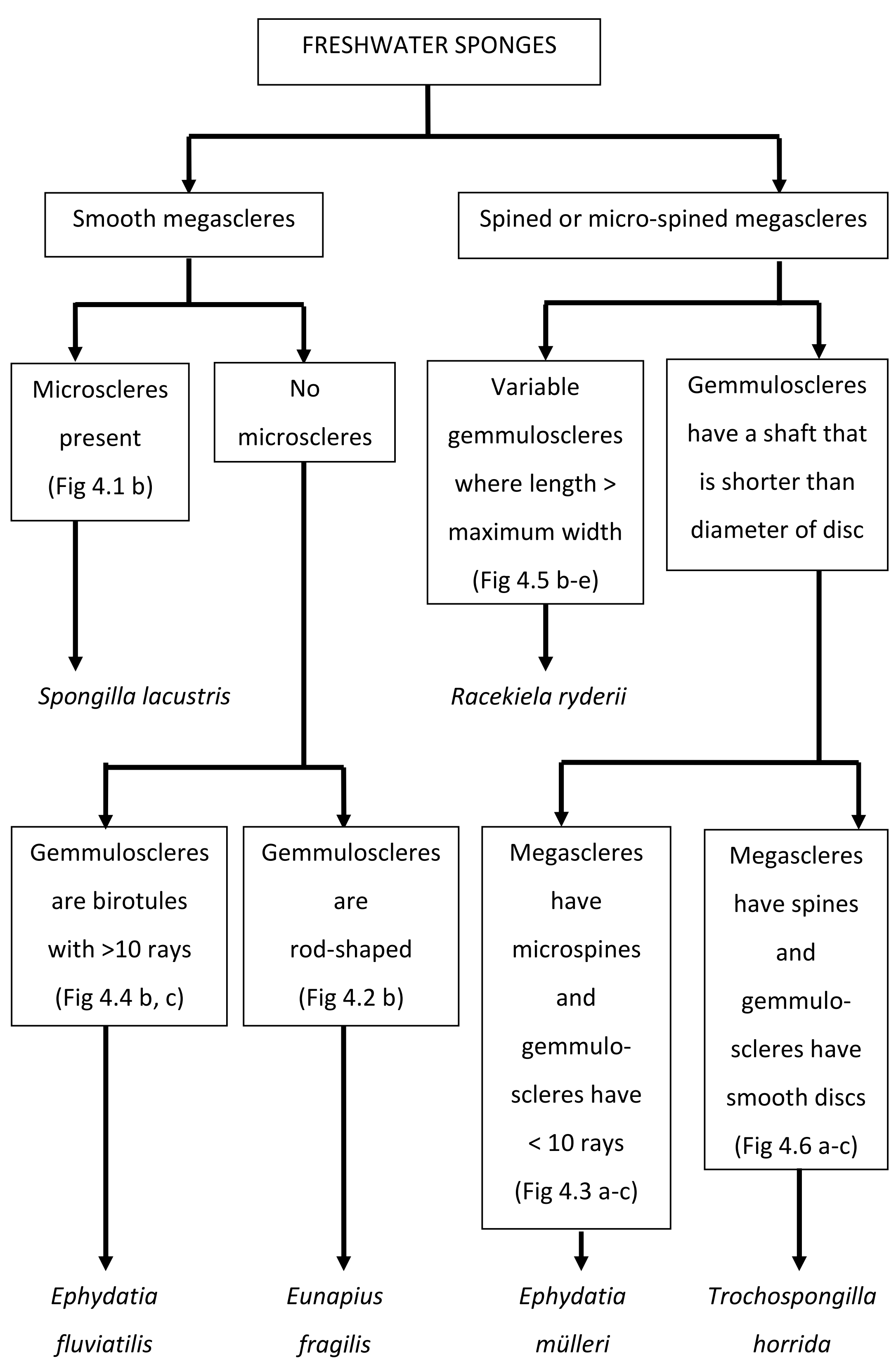

Fig.2. Key to species of freshwater sponges found in the UK, using megasclere morphology as an initial determinant.

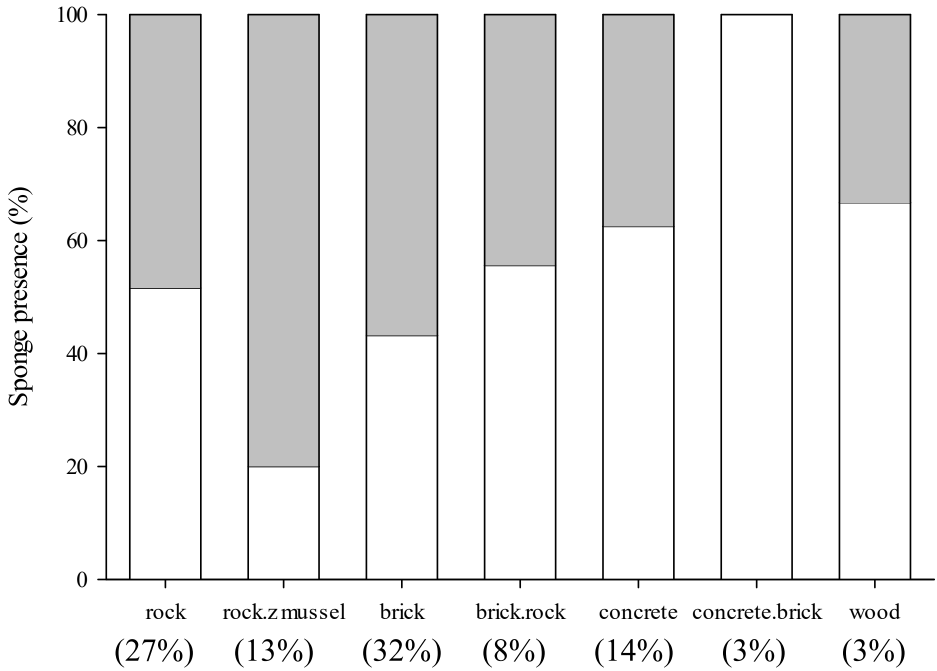

Fig.3. Sponge presence (white) on substrate types (rock; rock and zebra mussel; brick; brick and rock; concrete; concrete and brick; wood with other materials) at sites in Canal Survey data. Substrate occurrence as percentage shown in brackets. On average, sponges were absent from 51% of sites.

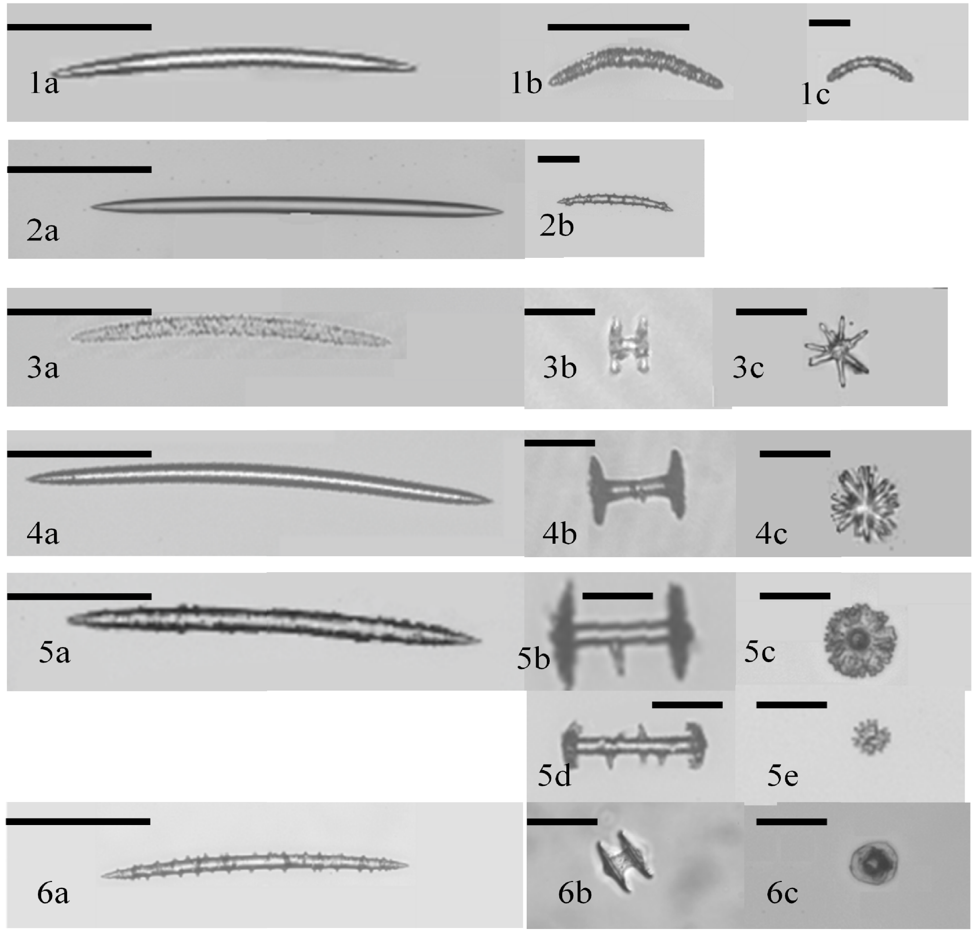

Fig.4. UK freshwater sponge spicules with digital light microscope images of megasclere, microsclere and gemmulosclere spicules. 1, *Spongilla lacustris*: a, megasclere, b, microsclere (scale bar = 50 µm), c, gemmulosclere. 2, *Eunapius fragilis*: a, megasclere, b, gemmulosclere. 3, *Ephydatia mülleri*: a, megasclere, b, gemmulosclere, c, rotule. 4, *Ephydatia fluviatilis*: a, megasclere, b, gemmulosclere, c, rotule. 5, *Racekiela ryderii*: a, megasclere, b, gemmulosclere, c, rotule, d, pseudo-gemmulosclere scale bar = 20 µm, e, rotule. 6, *Trochospongilla horrida*: a, megasclere, b, gemmulosclere, c, rotule. Scale bar for all megascleres = 100µm. Scale bar for all gemmuloscleres and rotules = 20µm.

```
                       10        20        30        40
                ....|....|....|....|....|....|....|....|....|
E.fluviatilis   CCAAGGAGTGCAACATGCGCGCGAGTCTTTGGGTGAGACGAAAAG
E.muelleri      .............................................
Eu.fragilis     ..................C..........................
S.lacustris     .............................................
R.ryderii       .............................................

                   50        60        70        80        90
                ....|....|....|....|....|....|....|....|....|
E.fluviatilis   CCCTGTGGCGCAATGAAAGTGAAGCGTCGGCTTGCCGACGCGAGG
E.muelleri      .............................................
Eu.fragilis     ......................................T......
S.lacustris     ..........................C..........G.......
R.ryderii       .....................................G.......

                       100       110       120       130
                ....|....|....|....|....|....|....|....|....|
E.fluviatilis   CGAGAGCCCTCTTCGCGGGGGCCCATCGTCGACCGATCCTATTCA
E.muelleri      .........CTC...G...T.........................
Eu.fragilis     .......A.C.CC.--...T.........................
S.lacustris     .........CTCA.--...T.........................
R.ryderii       .........CTCG.--...T.........................

                  140       150       160       170       180
                ....|....|....|....|....|....|....|....|....|
E.fluviatilis   CTTGTGAAGGGATTCGAGTGAGAGCGTGCCTGTTGCGACCCGAAA
E.muelleri      .............................................
Eu.fragilis     .............................................
S.lacustris     .............................................
R.ryderii       .............................................

                       190       200       210       220
                ....|....|....|....|....|....|....|....|....|
E.fluviatilis   GATGGTGAACTATGCCTGAGTAGGGTGAAGCCAGAGGAAACTCTG
E.muelleri      .............................................
Eu.fragilis     .............................................
S.lacustris     .............................................
R.ryderii       .............................................

                  230       240       250       260       270
                ....|....|....|....|....|....|....|....|....|
E.fluviatilis   GTGGAAGCTCGTAGCGATTCTGACGTGCAAATCGATCGTCAAACT
E.muelleri      .............................................
Eu.fragilis     .............................................
S.lacustris     .............................................
R.ryderii       .............................................

                       280       290       300
                ....|....|....|....|....|....|...
E.fluviatilis   TGGGTATAGGGGCGAAAGACTAATCGAACCATC
E.muelleri      .................................
Eu.fragilis     .................................
S.lacustris     .................................
R.ryderii       .................................
```

Fig.5. Alignment of the extended 28S rDNA D3 domains from *E. fluviatilis*, *E. mülleri*, *Eu. fragilis*, *S. lacustris* and *R. ryderii*. Identities have been indicated by dots and gaps.

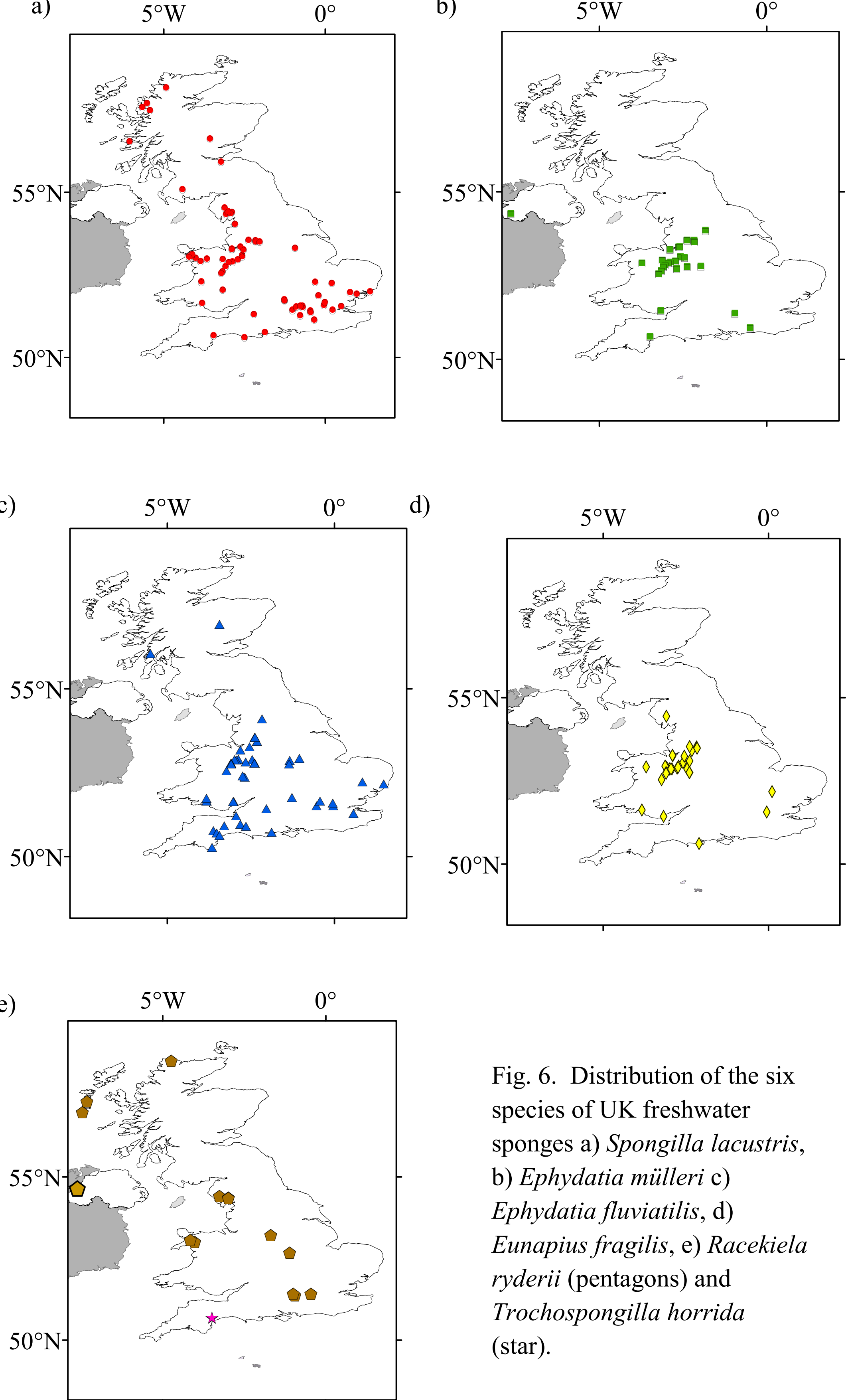

Fig. 6. Distribution of the six species of UK freshwater sponges a) *Spongilla lacustris*, b) *Ephydatia mülleri* c) *Ephydatia fluviatilis*, d) *Eunapius fragilis*, e) *Racekiela ryderii* (pentagons) and *Trochospongilla horrida* (star).

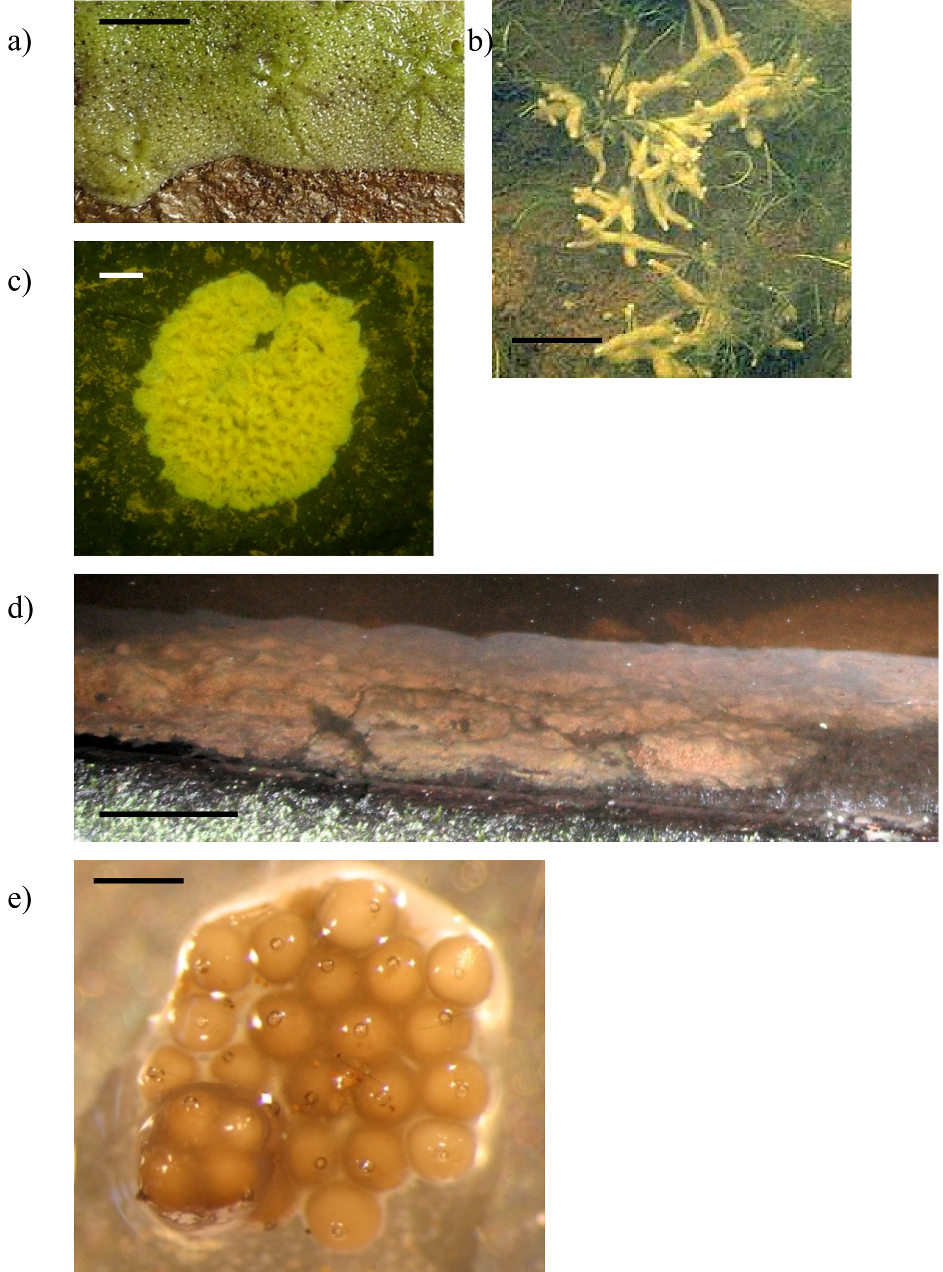

Fig.7. Examples of sponges found in the UK a) *S. lacustris* with astrorhiza surface (scale bar=0.01m) b) *S. lacustris* with finger-like growth form (scale bar=0. 1m) c) *E. fluviatilis* colony on stone (scale bar=0.01m) d) *E. mülleri* covering large area of vertical substrate (scale bar=0. 1m) e) Gemmular arrangement of *Eu. fragilis* showing “carpet” formation with foramina facing upwards, and a group of four gemmules (lower left) with foramina facing away from the cluster (scale bar=1mm).

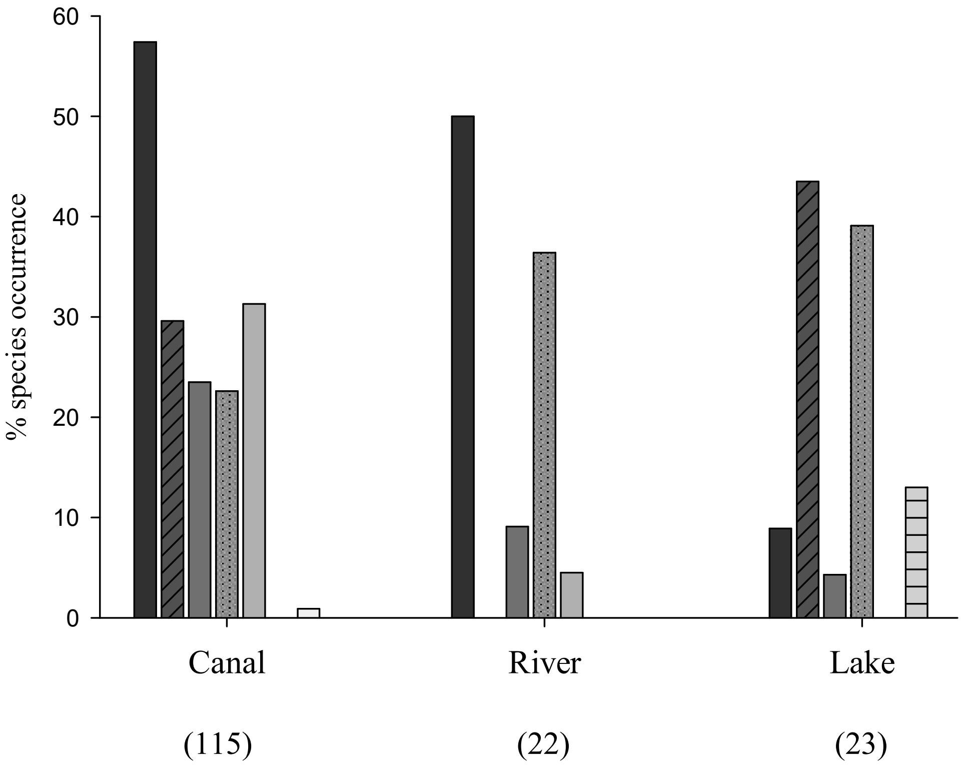

Fig.8. Sponge species presence at canal, river or lake habitats, with bars representing percentage of sites where species were present. Species were recorded alone at a site or co-occurring with other species. Number of habitat sites sampled shown in brackets. Key: ■ = sponge absence, ▨ = *S. lacustris*, ■ = *E. mülleri*, ▩ = *E. fluviatilis*, ■ = *Eu. fragilis*, ▤ = *R. ryderii*, □ = *T. horrida*.

Supporting Information 2.1.

Table S1. Types of substrate examined for the presence of sponges in Canal Survey data.

Substrate type	Occurrence in canal waterways
Brick	Used for original bridge construction or bank reinforcements.
Rock	Used for original bridge construction or bank reinforcements.
Concrete or breeze block	Used in construction of bridge footings and for waterway repairs
Metal or wire mesh	Used for reinforcement of waterway banks near locks and bridges.
Wood or macrophytes	Wood pillars used in bank reinforcements. Macrophytes found along canal edges in some areas, and in newly restored areas.

Table S2. Significance of water parameter variables recorded in the present study.

Variables recorded at 0.4m	Expected range	General significance to sponges - from Harrison (1974) unless indicated.	Significance to UK sponge species - from Harrison (1974) unless indicated.
Mean water Temp-erature (°C)	2 - 20	May influences sponge life cycle, morphological features or be a limiting factor for growth.	Low temperatures inhibit gemmule development in *S. lacustris*. *E. mülleri* is less tolerant than *S. lacustris* of lower temperatures (Økland and Økland, 1996).
Mean Cond-uctivity (μScm^{-1})	60 - 1200	Index of dissolved inorganic matter. A limiting factor at extremes for some sponge species. Low conductivity levels can inhibit skeletal spicule formation.	*S. lacustris* and *Eu. fragilis* occurs more frequently at areas with low conductivity <100 μS/cm (Paduano and Fell, 1997; Økland and Økland, 1996).
Mean water salinity (ppt)	0.03 - 0.6	Freshwater has salinity <0.5ppt, brackish water has salinity of 0.5 – 30ppt. Euryhaline species may be able to out-compete other species in stretches of canals near to the port with higher values.	High levels (>0.6ppt) of sodium (Na^{2+})has negative impact on growth rate of *E. fluviatilis* (Belas *et al*., 1989). The highest level recorded value for *E. fluviatilis* is 5.00 ppt (Tendal, 1967).
Mean pH	6 - 10	Low values of pH inhibit gemmule hatching. High values inhibit skeletal spicule formation in some species.	*Eu. fragilis* and *S. lacustris* tolerant to wider range of pH than other species. However, *S. lacustris* occurs more frequently at areas with low pH of 6.0 -6.7 (Paduano and Fell, 1997; Økland and Økland, 1996).
Mean dissolved oxygen ($mg\ l^{-1}$)	4 – 14.5	Oxygen is required for aerobic respiration. Oxygen levels may drop suddenly in areas with pollution or stagnant water.	*Eu. fragilis* and *E. mülleri* tolerant of low oxygen levels.
Mean value of total dissolved solids (TDS) ($g\ l^{-1}$)	0.1 - 0.9	Particles of dissolved solids may clog filtering system of sponge especially when growing on vertical substrate. High levels of TDS may prevent development from gemmules.	*Eu. fragilis* is tolerant to higher values of TDS than other species.
Oxidation Reduction Potential (ORP) (mV).	-30 - +100	Measures presence of reducing substances and a water system's capacity to either release or gain electrons in chemical reactions. Together with pH it controls cellular processes (Williams and Fulthorpe, 2003).	No specific data for sponges, but it has been suggested to be an important variable when assessing freshwater invertebrate communities (Williams and Fulthorpe, 2003).

Table S3. Canal Survey sites along canals, with bridge name. Latitude and longitude also shown.

Point	Lat	Long	Canal Name	Site Name
1	52.803	-2.301	Shropshire Union Canal	Norbury Junction
2	52.844	-2.400	Shropshire Union Canal	Park Heath Bridge
3	52.847	-2.416	Shropshire Union Canal	Soundley Bridge
4	52.851	-2.422	Shropshire Union Canal	Fox Bridge
5	52.861	-2.439	Shropshire Union Canal	Goldstone Bridge
6	52.887	-2.459	Shropshire Union Canal	Tyrley Bridge
7	52.901	-2.482	Shropshire Union Canal	Market Drayton
8	52.913	-2.478	Shropshire Union Canal	Victoria Bridge
9	52.958	-2.495	Shropshire Union Canal	Hawsmoor Bridge
10	52.975	-2.510	Shropshire Union Canal	Bagley Lane Bridge
11	53.043	-2.541	Shropshire Union Canal	Baddington Bridge
12	53.105	-2.574	Shropshire Union Canal	Bremilow Bridge
13	53.139	-2.756	Shropshire Union Canal	Crows Nest Bridge
14	53.150	-2.778	Shropshire Union Canal	Golden Nook Bridge
15	53.172	-2.816	Shropshire Union Canal	Egg Bridge
16	53.282	-2.889	Shropshire Union Canal	Stanlow Bridge
17	53.286	-2.891	Shropshire Union Canal	Powells Bridge
18	52.906	-2.757	Prees Branch Canal	Starks Lift
19	52.903	-2.756	Prees Branch Canal	Dobsons Bridge
20	53.036	-2.588	Llangollen Canal	Swanley Bridge
21	53.069	-2.575	Llangollen Canal	Wrenbury Heath Bridge
22	53.028	-2.613	Llangollen Canal	Wrenbury Church Lift Bridge
23	53.007	-2.667	Llangollen Canal	Marbury Church Bridge
24	53.730	-2.686	Llangollen Canal	Quoisley
25	52.982	-2.710	Llangollen Canal	Grindley Bridge
26	52.971	-2.708	Llangollen Canal	Whitchurch
27	52.968	-2.707	Llangollen Canal	Chemistry Bridge
28	52.952	-2.72	Llangollen Canal	Duddleston Bridge
29	52.949	-2.726	Llangollen Canal	Canbrian Railway Bridge
30	52.947	-2.723	Llangollen Canal	Blackoe Bridge
31	52.923	-2.729	Llangollen Canal	Platt Lane Bridge
32	52.914	-2.805	Llangollen Canal	Bettisfield Bridge
33	52.904	-2.818	Llangollen Canal	Hampton Bank Bridge
34	52.902	-2.831	Llangollen Canal	Lyneal Lane Bridge

35	52.896	-2.835	Llangollen Canal	Little Mill Bridge
36	52.900	-2.876	Llangollen Canal	Ellesmere Tunnel
37	52.894	-2.913	Llangollen Canal	White Mill Bridge
38	52.880	-2.936	Llangollen Canal	Peters Bridge
39	52.890	-2.958	Llangollen Canal	Maestermyn House Bridge
40	52.890	-2.990	Llangollen Canal	Hindford Bridge
41	52.920	-3.046	Llangollen Canal	Gledrid Bridge
42	52.927	-3.055	Llangollen Canal	Monks Bridge
43	52.930	3.070	Llangollen Canal	Chirk Tunnel
44	52.957	-3.065	Llangollen Canal	Whitehouse Bridge
45	52.959	-3.064	Llangollen Canal	Irish Bridge
46	52.970	-3.120	Llangollen Canal	Plas Ifan Bridge
47	52.970	-3.140	Llangollen Canal	Wenffrwd Bridge
48	52.980	-3.180	Llangollen Canal	Pentrefelin Bridge
49	52.880	-2.937	Montgomery Canal	GP Lock
50	52.818	-3.020	Montgomery Canal	Maesbury Marsh Bridge
51	52.817	-3.033	Montgomery Canal	Gronwyn Bridge
52	52.780	3.090	Montgomery Canal	Llanymynech Bridge
53	52.780	-3.095	Montgomery Canal	Walls Bridge
54	52.770	-3.110	Montgomery Canal	Carreghofa Locks
55	52.760	-3.090	Montgomery Canal	Four Crosses Bridge
56	52.719	-3.119	Montgomery Canal	Bank Lock
57	52.648	-3.150	Montgomery Canal	Whitehouse Mill Bridge
58	52.632	-3.169	Montgomery Canal	Sweeps Bridge
59	52.612	-3.184	Montgomery Canal	Brithdir Bridge
60	52.586	-3.192	Montgomery Canal	Garthmyl Bridge
61	52.578	-3.198	Montgomery Canal	Nr. Garthmyl Aqueduct
62	52.560	-3.229	Montgomery Canal	Glanhafren Bridge
63	52.772	-2.380	Newport Canal	Newport Central
64	52.771	-2.384	Newport Canal	Newport Channel
65	52.772	-2.389	Newport Canal	Tickethouse Lock
66	53.354	-2.632	Bridgewater Canal	Golborne Bridge
67	53.362	-2.602	Bridgewater Canal	Grappenhall,Walton
68	53.367	-2.579	Bridgewater Canal	London Road, Grappenhall
69	53.373	-2.549	Bridgewater Canal	Grappenhall, Lymm
70	53.431	-2.312	Bridgewater Canal	Whites Bridge
71	53.451	-2.301	Bridgewater Canal	Trafford Park
72	53.552	-2.169	Rochdale Canal	Oldham Road Bridge

73	53.549	-2.169	Rochdale Canal	Chadderton Bridge
74	53.527	-2.160	Rochdale Canal	White Gate Bridge
75	53.509	-2.158	Rochdale Canal	Failsworth Railway Bridge
76	53.130	-2.374	Trent and Mersey Canal	Wheelock Bridge
77	53.171	-2.418	Trent and Mersey Canal	Sandbach Bridge
78	53.200	-2.457	Trent and Mersey Canal	Middlewich Bridge
79	53.270	-2.539	Trent and Mersey Canal	Soote Bridge, Anderton
80	53.480	-2.264	Manchester Bolton and Bury	Middlewood Lock
81	53.479	-2.262	Manchester Bolton and Bury	Margaret Fletcher Lock
82	53.478	-2.259	Manchester Bolton and Bury	River Lock
83	53.558	-2.380	Manchester Bolton and Bury	Farnworth
84	53.554	-2.375	Manchester Bolton and Bury	Near Bridge14
85	53.556	-2.378	Manchester Bolton and Bury	Opposite.Carlisle Close
86	53.560	-2.387	Manchester Bolton and Bury	Near Hall Lane
87	51.652	-3.816	Neath and Tennant Canal	Crown Food, Metal Box
88	51.650	-3.820	Neath and Tennant Canal	Tricks Bridge
89	51.647	-3.825	Neath and Tennant Canal	Neath Junction Rail Bridge
90	51.694	-3.898	Swansea Canal	Pont John Bridge
91	51.737	-3.824	Swansea Canal	Ynysmeudwy Bridge
92	51.742	-3.814	Swansea Canal	Cilmaengwyn Bridge
93	51.659	-3.826	Swansea Canal	Crown Bridge
94	52.974	-3.139	Monmouthshire and Brecon	Sebastopol, Panteg Bridge
95	51.762	-1.270	Oxford Canal	Oxford Canal Road Bridge
96	53.949	-2.016	Leeds and Liverpool Canal	Horse Close Bridge
97	53.940	-2.012	Leeds and Liverpool Canal	Snaygill Stone Bridge
98	53.115	-2.548	Middlewich Canal	Cholmondeston Bridge
99	53.142	-2.493	Middlewich Canal	Church Minshall Bridge
100	50.689	-3.488	Exeter Canal	Countess Wear Bridge

Table S4. UK Survey sites with habitat type and site name. Latitude and longitude also shown.

Point	Lat	Long	Habitat type	Site
1	52.716	-2.694	Canal	Kiln Bridge, Shrewsbury
2	52.694	-2.676	Canal	Atcham, Shropshire
3	54.044	-2.801	Canal	Lancaster Canal
4	51.432	-0.326	Canal	Teddington
5	51.510	-0.538	Canal	GUC Canal Iver
6	51.513	-0.036	Canal	Regent Canal, London
7	51.655	-0.425	Canal	GUC Rickmansworth
8	51.762	-1.270	Canal	Walton Well
9	52.924	-1.051	Canal	Cotgrave
10	51.319	-2.210	Canal	Hiperton Marina
11	51.642	-3.027	Canal	Ty Coch Cwmbran
12	52.927	-3.055	Canal	Monks Bridge
13	52.842	-2.965	Canal	Heath House Bridge
14	52.839	-2.972	Canal	Corbett's Bridge
15	52.834	-2.982	Canal	A5 Bridge, Queen's Head, Oswestry
16	55.927	-3. 233	Canal	Union Canal, Edinburgh
17	52.615	-2.766	River	Cound Brook, Longnor
18	52.634	-2.774	River	Cound Brook, Stapleton
19	52.487	-2.777	River	Byne Brook, Wolverton
20	52.497	-2.763	River	Eaton Brook, Harton
21	52.497	-2.754	River	Eaton Brook, New Hall Farm
22	52.506	-2.739	River	Eaton Brook, Eaton
23	52.372	-2.723	River	Linney Ludlow
24	52.366	-2.684	River	Ledwyche Brook
25	52.405	-2.744	River	River Corve Stanton Lacy
26	52.373	-2.721	River	River Corve Ludlow
27	52.474	-2.816	River	Quinny Brook Craven Arms
28	52.873	-2.664	River	Soulton Brook
29	52.836	-2.673	River	River Roden, Lee Brockhurst
30	52.817	-2.657	River	River Roden, Moreton View
31	52.801	-2.634	River	River Roden, Moreton Mill Farm
32	52.867	-1.336	River	Trent Bridge
33	52.757	-1.358	River	Stream in Grace Dieu Wood
34	50.780	-3.624	River	River Creedy

35	50.914	-3.290	River	River Culm
36	52.170	1.472	River	Langham Bridge River Alde
37	52.872	-3.733	River	Afon Lliw Llanwchllyn
38	52.948	-3.688	River	Afon Tryweryn Frongoch
39	52.782	-1.971	River	River Trent
40	51.208	-2.939	River	Bason Bridge Somerset
41	52.984	-3.665	Lake	Llyn Hesgyn
42	52.916	-3.870	Lake	Llyn Hiraethlyn
43	53.053	-4.224	Lake	Llyn Nantlle Uchaf
44	53.015	-4.016	Lake	Llyn Llagi
45	53.072	-4.151	Lake	Llyn Cwellyn
46	53.126	-4.129	Lake	Llyn Padarn
47	51.640	-3.006	Lake	Cwmbran boating lake
48	56.058	-5.499	Lake	Dunardry Burn
49	56.914	-3.441	Lake	Baddoch Burn
50	55.093	-4.431	Lake	Round Loch of Glenhead
51	57.491	-5.431	Lake	Fionnaraich
52	54.543	-7.989	Lake	Mallybreen Lough
53	54.100	-2.165	Lake	Malham Tarn
54	54.430	-3.262	Lake	Burnmoor Tarn
55	50.276	-3.655	Lake	Slapton Ley
56	52.291	-0.315	Lake	Grafham Water
57	51.437	-1.018	Lake	Holybrook
58	51.729	-1.258	Lake	Oxford
59	50.963	-2.811	Lake	Lambrook
60	51.432	-2.031	Lake	Calne
61	51.929	0.978	Reservoir	Ardleigh Reservoir
62	50.900	-2.639	Reservoir	Sutton Bingham Reservoir
63	53.052	-2.688	Mere	Deer Park Mere
64	53.048	-2.460	Moss	Wybunbury Moss
65	52.594	1.492	Broad	Buckenham Hassingham
66	52.716	1.461	Broad	Burntfen Broad
67	52.723	1.606	Broad	Heigham Sound
68	52.665	1.537	Broad	Upton Little Broad
69	52.723	1.515	Broad	Cromes.Broad
70	51.462	-3.166	Brackish	Cardiff Bay Wall

Table S5. Natural History Museum data with UK specimen sites, habitat type, latitude and longitude.

Point	Lat	Long	Habitat Type	Site Name
1	54.343	-3.071	Lake	Coniston Water, Lake District
2	54.372	-2.991	Lake	Priest's Pot, Lake District
3	50.609	-2.501	Lake	Little Sea Lagoon, Dorset
4	50.709	-3.526	Lake	Salmon Pool, Exeter
5	52.229	0.839	Lake	Drinkstone Park, Suffolk
6	57.229	-7.347	Lake	Grogam Uist
7	56.969	-7.494	Lake	Barra Outer Hebrides
8	51.590	-0.046	Lake	Walthamstow Waterworks
9	56.549	-6.066	Lake	Isle of Mull Loch Achanalt Ross
10	54.351	-7.647	Lake	Lough Erne, Enniskillen
11	51.417	-0.460	Lake	Conduit Littleton Reservoir
12	51.878	-0.210	Lake	Knebworth Park, Herts
13	54.363	-2.986	Lake	Esthwaite, Lake District
14	54.410	-2.890	Lake	Bletham Beach, Pullwoods, Windermere
15	54.469	-3.087	Lake	Codale Tarn, Lake District
16	50.649	-2.093	Lake	Little Pea Lagoon, Furzbrook, Dorset
17	52.262	0.203	Lake	Mere Fen Road, Waterbeach
18	56.632	-3.577	Lake	Loch Ordie, Perth
19	54.535	-3.122	Lake	Watendlath Tarn, Lake District
20	54.430	-3.010	Lake	Loughrigg Tarn, Lake District
21	51.145	-0.347	Lake	Vann Lake Ockley Surrey
22	54.374	-2.938	Lake	Windermere, Lake District
23	57.710	-5.531	Lake	Loch Maree Rosshire
24	51.554	0.495	Lake	Fish Farm Tanks, Haselmere
25	58.178	-4.937	Lake	Loch Fleodach Coire
26	57.579	-5.669	Lake	Loch Mhullaid Upper Diabraig, Rosshire
27	51.437	0.223	Lake	Brooklands Lake, Dartford, Kent
28	51.972	0.774	River	River Stour At Burs, Suffolk
29	52.199	0.114	River	Cam backwater, Newnham Mill
30	50.764	-1.872	River	River Stour, Near Bournemouth
31	50.634	-3.438	River	River Exe, Near Exeter
32	50.949	-0.502	River	River Arun, Near Pulborough
33	51.993	1.390	River	River Deben, Suffolk
34	51.536	-0.900	River	Henley-on-Thames
35	51.568	-0.712	River	Thames Cookham
36	51.523	-0.702	River	Thames Near Maidenhead
37	51.382	-0.456	River	Thames Weybridge
38	51.567	-0.773	River	Marlow Lock, Thames
39	52.054	-3.178	River	River Wye
40	51.380	-0.954	River	Swallowfield, River Blackwater, Reading
41	53.221	-1.687	River	Peak District
42	53.319	-0.941	Canal	Under Canal Bridge opposite Topshun
43	51.762	-1.270	Canal	Oxford Canal Rugby
44	53.848	-1.830	Canal	Leeds and Bradford Canal, Bingley, stretch 18
45	52.686	-1.102	Canal	GUC Leicester Line
46	51.267	-0.781	Canal	Basingstoke Canal, Surrey
47	53.507	-2.039	Canal	Huddersfield North Canal Lock 12
48	50.668	-3.468	Canal	Turf Inn Lock Exeter Canal
49	53.319	-0.941	Canal	Retford Town Lock, Chesterfield Canal
50	52.766	1.520	Broad	Stalham Broad Norfolk

Table S6. Variation in megasclere spicule dimensions of UK freshwater sponge species with mean, standard error (S.E.), standard deviation (S.D.) (n>25 of each spicule type except *T. horrida* where n>12).

Species	Spicule type	Length (µm)			Width (µm)			Rotule diameter (µm)		
		x̄	S.E.	S.D.	x̄	S.E.	S.D.	x̄	S.E.	S.D
Spongilla lacustris	Megascleres	235	2.0	24.8	9	0.2	2.0	—	—	—
	Microsclere	71.2	1.1	9.5	3.9	0.1	1.2	—	—	—
	Gemmulosclere	65.5	2.6	14.8	5.4	0.3	1.5	—	—	—
Ephydatia mülleri	Megascleres	222	3. 9	27.1	10	0.4	2.8	—	—	—
	Gemmulosclere	10.8	0.2	1.5	3.6	0.2	1.6	17.5	0.2	1.9
Ephydatia fluviatilis	Megascleres	315	5. 9	40.1	11	0.3	2.4	—	—	—
	Gemmulosclere	27.1	0.5	2.3	4.0	0.1	0.9	19.7	0.2	1.8
Eunapius fragilis	Megascleres	267	5.7	18.3	11	0.6	2.4	—	—	—
	Gemmulosclere	72.1	1.7	8.2	5.5	0.2	1.1	—	—	—
Racekiela ryderii	Megascleres	281	5.7	46.3	8.4	0.3	2.3	—	—	—
	Gemmulosclere	41.1	0.9	4.3	2.8	0.1	0.8	20.1	0.3	2.0
	Pseudo gemmulosclere	44.3	4.1	8.4	3.7	0.3	0.6	13.1	0.3	0.8
Trochospongilla horrida	Megascleres	202	3.8	13.1	8.5	0.2	0.8	—	—	—
	Gemmulosclere	10.8	0.7	2.9	3.8	0.2	0.8	12.8	0.5	1.8

Table S7. Range and average values of water factors (temperature, conductivity, total dissolved solids, salinity, pH, oxidation reduction potential) with standard error (S.E.) for four freshwater sponge species found in the UK.

		Spongilla lacustris	*Ephydatia müllerі*	*Ephydatia fluviatilis*	*Eunapius fragilis*
Temp (°C)	x̄±S.E.	11.97±0.41	12.5±0.45	12.43±0.58	12.35±0.49
	Range	7.18 -18.12	9.34 – 16.00	7.86 – 18.12	7.18 – 18.12
Cond (μScm^{-1})	x̄±S.E.	180.48±24.04	195.32±34.60	322.93±55.32	233.42±41.73
	Range	60.00 -687.67	68.50 – 921.67	79.83 – 921.67	61.00 – 921.67
TDS ($g\ l^{-1}$)	x̄±S.E.	0.16±0.02	0.16±0.03	0.27±0.05	0.20±0.04
	Range	0.05 – 0.67	0.05 – 0.74	0.07 – 0.74	0.05 – 0.74
Salinity (ppt)	x̄±S.E.	0.12±0.02	0.12±0.02	0.22±0.04	0.15±0.03
	Range	0.04 - 0.46	0.04 – 0.57	0.05 – 0.57	0.04 – 0.57
Oxygen ($mg\ l^{-1}$)	x̄±S.E.	11.06±0.49	10.19±0.55	9.83±0.64	11.26±0.62
	Range	4.91 – 15.6	4.88 – 15.60	4.84 – 14.73	4.84 – 17.97
pH	x̄±S.E.	7.60±0.08	7.63±0.10	7.64±0.09	7.65±0.08
	Range	6.75 – 8.87	6.12 – 8.87	6.77 – 8.48	6.77 – 8.48
ORP (mV)	x̄±S.E.	4.71±3.47	6.20±5.67	-5.09±6.73	-2.70±5.20
	Range	-19.05 – 47.07	-53.23 - 94.10	-64.80 – 47.07	-64.80 – 47.07

Chapter 3. Salinity and silicon levels determine freshwater sponges (Porifera, Demospongiae) summer abundance in anthropogenic microhabitats.

Summary

Differing tolerance ranges of freshwater sponge species have been suggested with regard to values of habitat water parameters. Wide variations in water parameters were recorded in UK anthropogenic microhabitats. The effects of twelve water parameters on the summer abundance of four UK freshwater sponge species (*Spongilla lacustris*, *Ephydatia mülleri*, *E. fluviatilis*, *Eunapius fragilis*) in anthropogenic microhabitats were investigated. Although, water parameters such as salinity and silicon regulate aspects of cell metabolism, sponge species had differing responses. A glm analysis revealed that salinity was linked with *S. lacustris* and *E.mülleri* colony abundance, and silicon was linked with *E. fluviatilis* and *Eu. fragilis* colony abundance. This information is applicable when interpreting the roles of specific water parameters in influencing freshwater sponge distribution.

Introduction

Freshwater sponge colonies inhabit nearly all types of freshwater environments and can comprise a major component of the benthic community (Reiswig *et al*., 2009). As well as adding biodiversity, sponges are important components of a freshwater habitat as a result of their filtering activity (Frost, 1978, Reiswig, 1971), their ability to support a diverse assemblage of epibionts (Matteson, and Zacobi, 1980; Kamaltynov *et al*., 1993), and in the biological control of invasive species such as zebra mussels (*Dreissena polymorpha*) (Garton and Haag, 1993; Ricciardi *et al*., 1998). However, freshwater sponges are typically overlooked in surveys and remain an understudied group (Reiswig *et al*., 2009). The present study addresses questions linking UK freshwater sponge assemblages to tolerances to water parameters. Practical demarcation values of key water parameters will assist modelling of population changes of these important freshwater invertebrates.

Five species of freshwater sponge have been reported in the UK (Pronzato and Manconi, 2001): *Spongilla lacustris* (Linnaeus, 1759), *Ephydatia mülleri* (Lieberkühn, 1856), *Ephydatia fluviatilis* (Linnaeus, 1759), *Eunapius fragilis* (Leidy,

1851) and *Racekiela ryderii* (Potts, 1882)(see Chapter1). *Spongilla lacustris*, *E. mülleri*, and *T. horrida* are holartic species, while *E. fluviatilis* and *Eu. fragilis* are both apparently cosmopolitan in their distributions; *R. ryderii* has an unusual amphiatlantic distribution (see Chapter 6). The annual pattern life history for these species in the UK involves growth from gemmules in the spring, continued size increase throughout the summer, followed by tissue regression and gemmulation in autumn and winter (Jewell 1939; Harrison 1974; Poirrier 1974; Pennak, 1989; Reiswig *et al*., 2009). Maximum size of encrusting sponge colonies occurs during the summer (Manconi and Pronzato, 1991). However, it is not known how summer sponge abundance is affected by habitat conditions or if conditions affect all species in the same way.

Sponges require habitats with a stable substrate and an adequate supply of particulate food (Reiswig *et al*., 2009). There are a few records of sponges located in UK rivers and lakes (Parfitt, 1868; Annandale, 1908; Mellanby, 1963; Waterson, 1981; Pronzato and Manconi, 2001). In addition to rivers and lakes, canals can serve as an important habitat for sponges have the potential to add to knowledge of sponge assemblages. However, in contrast to rivers and lakes, canals connect various drainage systems containing different inputs so that different canal sections may exhibit variations in water parameters. This potential variability makes canals an exceptional class of habitat, quite distinct from rivers and lakes (Hadfield, 1959; Willby and Eaton, 1996; Yorke, 2003). Also, following neglect and disuse of some canals certain stretches are now isolated from previously connected waterways (Hadfield, 1959; Russell, 1971; Yorke, 2003). Thus there are many potential factors that may influence UK freshwater sponge species in canal habitats.

Water parameters limit the distribution of zooplankton species and influence aquatic community structure (Tilman, 1988; Webster *et al*., 1992; Arnott and Vanni, 1993). However, it is unclear if the level of variation in canal water parameters is sufficient to affect sponge species abundance. If so, it is likely that not all sponge species will be affected in the same way. For example, physiological adaptation to ranges in water parameter values for cosmopolitan species (e.g. *Eu. fragilis* and *E. fluviatilis*), may be wider than those for species with smaller distributions (e.g. *S.*

lacustris and *E. mülleri*) (e.g. Cohet *et al*., 1980; Pronzato and Manconi, 2001) providing insight into species distribution patterns (Cohet *et al*., 1980).

Possible limiting macronutrients (silicon, nitrogen, phosphorus, and calcium), together with water parameters (temperature, conductivity, total dissolved solids (TDS), salinity, dissolved oxygen, pH, oxidation/reduction potential (ORP) and velocity) and canal fragmentation were considered in the present study. Among these parameters salinity and silicon would appear to be important to freshwater sponges. Salinity increases have been shown to decrease filtration activity and growth in some freshwater sponge species (Simpson, 1984). Also, silicon is needed for development and growth of spicule skeletal elements. Of the three categories of spicules (megascleres, microscleres, and gemmuloscleres) megascleres are the largest. They make up the main framework of a sponge and are found in all actively growing freshwater sponges (Reiswig *et al*., 2009). Megasclere morphology reveals species-specific diversity between some species (Reiswig *et al*., 2009), and this may correspond to silicon as a key parameter with low levels limiting sponge abundance.

The few studies to describe the water chemistry parameters that determine freshwater sponge abundance/presence suggest calcium may be important. However, such work is between 15 and 80 years old and either based on one species (Colby *et al*., 1999; Melão and Rocha, 1999) or a limited number of abiotic factors (Jewell, 1935, 1939). This study is the first to examine abundance of four species simultaneously, considers fragmented canals, and covers twelve water parameters that other researchers have suggested influence sponge abundance (Supporting Information 3.1, Table S1).

The paucity of information regarding freshwater sponge ecological requirements means that it is difficult to predict if sponge species are eurytopic and abundant at all available canal sites, or are stenotopic and are abundant at specific sites. To improve understanding of the factors affecting sponge species in canals, the current study investigated the abundance of four species (*S. lacustris*, *E. mülleri*, *Eu. fragilis*, *E. fluviatilis*) across twelve canal sites. With varying water inputs, it was expected that canal water parameters and habitat fragmentation had the potential to

determine sponge abundance; however, the scale and strength of the effects would depend on the requirements of the individual species, including their tolerance to seasonal changes. This work uses seasonal surveys of water parameters, spicule size, and sponge summer abundance to assess factors that influence sponge species in canals. ANOVA and cluster analyses were used to assess similarities in species' abundance. Generalised linear modelling methods (glm) were used to evaluate water parameters, and predict significant parameters. Non parametric tests were used on these significant parameters. The overall goal was to provide summer demarcation values of significant parameters associated with sponge abundance in canal habitats, contrasting the effects of seasonal parameter variability, species requirements, and canal ecology.

Methods

Preliminary analysis and sample sites

To ensure canal sites that encompassed a diversity of water parameters were sampled, preliminary surveys of the main canal waterways located in the north-west of the UK were conducted. This sampling included a number of connected and isolated canal stretches. To identify the sites that differed most in water parameters, cluster analysis of the environmental matrix of water parameters was conducted (SPSS v. 22, IBM Corp, 2013; Supporting Information 3.1, Fig. S1). Twelve sampling sites (Supporting Information 3.1, Fig S1) that occupied different clusters on the dendrogram were identified. Sites were separated by >20 km, and all were shaded. Nine sites were on connected stretches of canal, and three were located on canal sections that were isolated for ~60 years. Sampling sites were located on two replicate waterways, and each site had three sub-sites separated by 0.5 km to 10 km (Fig. 2).

Survey protocol and taxon identification

Twelve key water parameters (see Supporting Information 3.1, Table S1) were recorded bi-monthly at a depth of 0.4 m at every site from January to December 2012: temperature, conductivity, total dissolved solids (TDS), salinity, dissolved oxygen, pH, and oxidation-reduction potential (ORP) were measured in situ using a multi-parameter recorder (YSI 560; YSI Inc., Yellow Springs, OH, U.S.A.); a 0.1 l

water sample was taken for analyses of nitrate, phosphate, silicon and calcium following methods of Radojevic and Bashkin (2006); water velocity was measured using a floating ball (see Dodds, 2002). In July 2012, these parameters were recorded at all sub-sites. Additionally in July 2012, sub-sites were assessed for sponge presence, with the area of substrate occupied by each sponge colony recorded (to a depth of 0.4 m) using a transparent, plastic grid (following Dröscher and Waringer, 2007). Samples (~1 cm^3) of each colony were placed in 100% ethanol, and used to identify species.

Species were identified based on spicule morphometrics and meristics and were identified based on spicule characteristics following taxonomic keys of Pronzato and Manconi (2001) and Manconi and Pronzato (2002) (for a simplified version of these keys, see Chapter 2). Samples (~1 cm^3) were boiled in 100% nitric acid for 2 min and then left for ~24 hours, to separate spicules from the tissue. After three washes in water and re-suspension in 100% ethyl alcohol, spicules were transferred to slides. Spicules were examined at 200 times magnification; images were captured with a digital camera and analysed with an image analysis system. The minimum, maximum and mean (±SE) length and width of the spicules were determined from >50 spicules of each type from several specimens (following Volkmer-Ribeiro and de Souza Machado, 2007). To provide an index of silicon requirements, megasclere volumes were determined by approximating spicules as cylinders. To assess differences in spicule volume between species, mean megasclere volumes were compared by ANOVA, followed by a post-hoc Bonferroni test (Zar, 2010), and planned contrasts between species using SPSS.

Statistical analyses

Two analyses were used to assess similarities in species' abundance between sub-sites. First, ANOVA was used to compare species abundance between sub-sites. Second, the degree of sponge species co-existence among all sub-sites was assessed by cluster analysis using SPSS examining the overall Jaccard coefficient (J). Where $J = (a/[a + b + c])$, a is the number of species present at two sub-sites, and b and c are the total number of species occurring at each sub-site but not the other (see

Clifford and Stephenson, 1975; Plafkin *et al*. 1989). This was repeated to assess co-existence of species located at sub-sites on isolated canals.

Generalised Linear Modelling (glm)

To further explore the influence of fragmented canals and water parameters on sponge species abundance, multiple logistic regression was used in the R 'stats' package (R Core Team, 2014, v 2.12.0). Regressions were performed on each of the four species data sets used to record abundance. First, maximum, minimum, yearly average and seasonal averages of all parameters from sites were considered. Second, average water parameters from all sub-sites recorded in July 2012 were considered. In both cases, water parameters and predictor data not meeting the assumptions of normality and equal variances were *ln* transformed prior to glm analysis (Zar, 1999) using SPSS. To account for possible collinearity among water parameters recorded at sites and sub-sites, pairwise scatter plots were constructed using R, and covariants that were highly correlated ($R^2>0.5$) were dropped (Zuur *et al*., 2010). In a group of collinear variables the variable that was the most strongly correlated with the dependent variable was retained. The Cook statistic was assess using R, and outlier sub-sites with high values (>0.5) that may dominate glm results (Zuur *et al*., 2010) were removed. Glm were fitted using Gaussian family type and identity link function to correlate species' abundance and water parameters. Only data from sub-sites where sponges were present were included in glm analyses as there may be many reasons, unrelated to these data, why sponges were absent from a sub-site. Further assessment removed zero values of species abundance to simplify graphical interpretation of analyses (Zuur *et al*., 2010). All possible combination of water parameters were correlated with species' abundance. Only additive models were considered as including interaction terms would have lead to model fitting problems. The best models were considered to be those with the fewest predictors and those that received "substantial" support (i.e., ΔAIC <2; Burnham and Anderson, 2002) of the overall best model. Glm analyses using only average parameter values from sub-sites in July 2012 was also carried out, with virtually identical results (Supporting Information 3.1, Table S4).

Further analysis was focused on statistically significant parameters i.e. salinity and silicon (see Results Table 4). At sub-sites with sponges, a Mann-Whitney U test (data were not normally distributed) was conducted to evaluate whether abundances varied with a) salinity and b) silicon concentrations using SPSS. Sponge abundance was evaluated at high and low silicon values. Low values of silicon concentrations were considered to be <0.7 mgl^{-1} as this is the lowest tolerance for *E. mülleri* (Harrison, 1974). Species' abundance was evaluated in a similar way with salinity, where low values of salinity were considered to be below the yearly average value of 0.2 ppt.

Results

Water parameters

Average annual conductivity, salinity, silicon, ORP, phosphate, and nitrate varied by more than 25% of their overall means at >80% of sub-sites, indicating that this study assessed a wide range of these parameters (Table 1). By contrast, average annual water temperature, dissolved oxygen, and pH exhibited less variability (i.e. <25% of the overall mean). Over the study period, average values of TDS, salinity, dissolved oxygen, pH, ORP, nitrate, and phosphate were lower during summer months (Fig. 1). Fluctuations in silicon, phosphate, and nitrate at some sub-sites exhibited levels at or near zero (<0.1 mgl^{-1}). Average values of ORP were below zero (negative) in summer and autumn (Fig. 1b).

Sponge species and characteristics

Sponges were present at most (81%) sub-sites located at connected and isolated canal stretches (Fig. 2). The following sponges were identified: *Spongilla lacustris*, *Ephydatia mülleri*, *Eunapius fragilis*, and *Ephydatia fluviatilis*. Length and width ranges of the four sponge species were similar to those described elsewhere in their ranges (Table 2). Mean megasclere volume differed between the four species ($F_{4,245}$ =14.41, p <0.01) (Fig. 3). Post-hoc tests contrasting each species indicated *E. fluviatilis* and *Eu. fragilis* had significantly larger megascleres than *S. lacustris* and *E. mülleri* (p <0.01) (Supporting Information 3.1, Table S2).

Species frequency, abundance, and co-occurrence

Spongilla lacustris was the most abundant species, with large (>400 cm^2) substrate cover at some sub-sites (Fig. 4). Although *S. lacustris* colonies can form finger-like growth (Reiswig *et al*., 2009), only encrusting colonies were found on the vertical sides of the canal allowing consistency between species when recording sponge abundance. There was a significant difference in *S. lacustris* abundance between sub-sites (p_{35}<0.01) with colonies covering all available substrate at some sub-sites, and absent at others. The abundances of both *S. lacustris* and *E. mülleri* were largest at sub-sites where *E. fluviatilis* was absent (Fig. 2). *Eunapius fragilis* occurred most frequently at sub-sites (50%) (Fig. 4), but colonies tended to be smaller and covered less substrate than *S. lacustris* and/or *E. mülleri*.

As reported by other researchers (e.g. Jewell, 1935; Ricciardi. and Reiswig, 1993; Pronzato and Manconi, 2001), some species are frequently found co-existing with other species. Across all sub-sites, the most frequently coexisting pair of species at all sub-sites was *S. lacustris – E. mülleri*, (Jaccard similarity index J = 0.77), with *E. fluviatilis* often segregated (J = 0.07–0.10) (Table 3a). However, at sub-sites located on isolated stretches of canal, the most frequently coexisting pair of species was *E. fluviatilis – Eu. fragilis* (J =0.50) with *S. lacustris* occurring less often (J = 0.14–0.33) (Table 3b).

Habitat characteristics

The use of glm analysis was appropriate to quantify the relationship between count data (such as abundance) with multiple environmental factors (such as water parameters) (Crawley, 2005). However, analyses of maximum, minimum, average, and seasonal average parameter values from sites, indicated problems associated with collinearity. Values of temperature and dissolved oxygen remained in the model, following the removal of correlated factors. Summer data analyses from sub-sites indicated that four water parameters, conductivity, salinity, TDS, and calcium were correlated (R^2 >0.5) (Supporting Information 3.1, Fig. S2). To remove analytical problems associated with collinearity, salinity, TDS, and calcium were removed from the analyses (Zuur *et al*., 2010); this was based on the consideration that conductivity may include charged particles (including those of Na^+, Ca^{2+}, and Cl^-) (Radojevic and

Bashkin, 2006). To determine which parameters may be driving sponge species abundance, all remaining parameters were considered in the initial glm analysis. Subsequently, one or two outlier sub-sites were removed (Supporting Information 3.1, Table S2).

Best models with substantial support were considered (Supporting Information 3.1, Table S3) for each species, with summer values of silicon, maximum values of dissolved oxygen, and factors associated with conductivity, featuring most often. The best model contained either one or two significant parameters (Table 4); specifically, average summer silicon concentration was a significant factor for abundance models for *E. fluviatilis* and *Eu. fragilis*, with greater silicon concentrations in areas of greater abundance (Fig. 5). Average summer temperature or maximum temperature (recorded in the summer) was a significant positive parameter in abundance models for *S. lacustris* and *E. mülleri*. Although, for this species pair, greater levels of factors associated with summer conductivity had negative impacts. Average summer values of ORP were significant for abundance models of *E. fluviatilis* and also featured in models with substantial support (Supporting Information 3.1, Table S3) for *Eu. fragilis*. This negative impact (t =-2.98) suggests that a greater abundance of *E. fluviatilis* occurred at sub-sites with negative ORP values. Identical analyses on only average summer parameter values yielded virtually identical results.

Mann-Whitney U tests results were significantly higher for abundances of *S. lacustris* (z =-2.36, p<0.05) and *E. mülleri* (z=-2.08, p<0.05) at low summer salinity sub-sites (<0.20 ppt) (Fig. 6). The two other sponge species had significantly larger abundance (*E. fluviatilis*; z=2.25, p<0.05; *Eu. fragilis*; z=3.05, p<0.01) at high summer silicon (> 0.70 mgl^{-1}) sub-sites (Fig. 7).

Discussion

Although sponge assemblages increase invertebrate biodiversity (Manconi and Pronzato, 2008), and have other important roles such as bio-filtration in canals (see Chapter 1), their habitat tolerances have been examined for only relatively few parameters and not at all in the UK (e.g., Økland and Økland, 1996). What do UK

canal systems have to add to knowledge of sponge assemblages? This study has provided the first demonstration that a) the canals surveyed in the UK support a variety of sponge assemblages; b) canals have a sufficiently diverse environmental character to impact sponge distribution; c) that abundance of freshwater sponge species in UK canal habitats is closely related to specific water parameters values. The demarcation values of significant water parameters, i.e. salinity and silicon, have the potential to be used to manage freshwater habitats and develop water quality goals.

Many cosmopolitan species are tolerant of a wide range of conditions (e.g. Cohet *et al*., 1980). The frequent presence of *Eu. fragilis* at canal sub-sites reflects its occurrence across Europe (Pronzato and Manconi, 2001) and it indeed has cosmopolitan distribution. It was surprising, however, that the other apparently cosmopolitan species *E. fluviatilis* (Pronzato and Manconi, 2001), occurred less frequently at sub-sites. The regular absence of these cosmopolitan species at sub-sites suggests unsuitable conditions for colonisation /persistence or water parameters not considered here. For example, potentially important water parameters to impact sponge cell functioning include magnesium (Belas *et al*., 1989), copper, or zinc (Francis and Harrison, 1988).

Spongilla lacustris was located slightly less frequently (44% of sub-sites) than *Eu. fragilis* (50% of sub-sites), but with a significantly larger average abundance which were orders of magnitude greater than that of other species at some sub-sites. This may reflect an increased growth rate compared to other species at some sub-sites during the summer, and potentially dominate a benthic community (Ricciardi *et al*., 1995). *Spongilla lacustris* and *E. mülleri* both have a holartic distribution (Pronzato and Manconi, 2001), although *E. mülleri* is considered to have a patchy distribution pattern in Europe (Stephens, 1920; Tendal, 1967; Waterston, 1981). However, here *E. mülleri* was located at canal sub-sites with only a slightly lower frequency (39% of sub-sites) than *S. lacustris*, and its patchy distribution may be related to specific water parameter tolerances or factors not recorded here e.g. habitat disturbance (S. Leys pers comm).

Annual extremes of twelve water parameters, as well as seasonal averages were considered in glm analyses, as any of these factors were potentially important to abundance of freshwater sponge species. Water temperature (between 15 and 28°C) has been shown to be to be a key factor for bryozoans (Wood, 2001), and low pH an important limiting factor for crayfish (Hobbs & Lodge, 2001) and water mites (Smith *et al*., 2001) in freshwater. For sponges there are fewer data but Wisconsin surveys in North America suggest calcium is the main factors determining sponge presence (Jewell, 1939). Although conductivity was highlighted by glm analyses, note that the effect of conductivity can be driven by TDS, salinity or calcium (R^2>0.5). Conductivity measures dissolved cations (Na^+, Ca^{2+}) and anions (Cl^-) (Radojevic and Bashkin, 2006). TDS values also include ions together with any uncharged molecules, e.g. dissolved organic compounds (Dodds, 2002). Both conductivity and TDS values could potentially be driven by salinity (Na^+, Cl^-) or calcium ions (Ca^{2+}). However, it seems clear that seasonal variations in salinity exhibit a similar pattern to those of conductivity and TDS, and that this pattern is different to that of calcium concentrations (Fig. 1). Thus, it seems likely that values of conductivity and TDS are driven by salinity (Na^+, Cl^-) not calcium (Ca^{2+}). These results are further supported by salinity demarcation values for species' abundance (Fig. 6). The implication is that sponge species have different tolerances to ionic concentration, specifically to salinity (Na^+, Cl^-).

Salinity, together with silicon, ORP, and summer temperature have emerged as drivers of species' abundances for sponges at canal sub-sites. Temporal variation in temperature affected all sub-sites in a similar way. However, the spatial variation of salinity, silicon, and ORP is a feature of canal sub-sites in this study. Spatial variation in silicon and salinity factors is driven by groundwater and mineral weathering (Dodds, 2002; Radojevic and Bashkin, 2006) (Supporting Information 3.1, Table S1); while other factors such as temperature and ORP vary seasonally but in a more predictable way at all sites. Average values of salinity and silicon were lowest at all sub-sites in the summer (Fig. 1). Average values of ORP were more negative during the summer and at some canal sub-sites ORP values remained negative (a reducing environment) for most of the year (Supporting Information 3.1, Table S2). Varying temporal and spatial water parameters limit some invertebrates

by (Thorp and Covich, 1991), but here variations in salinity, silicon, ORP and summer temperature were used to answer question concerning abundance. Water parameters and utilisation by sponges could have an influence on the metabolism of the canal life and might exert a strong influence on other organisms such as the phytoplankton (Melão and Rocha, 1999).

Importance of salinity

Salinity is a key variable impacting distribution of many freshwater taxa (Rutherford and Kefford, 2005), including some sponge species e.g. *E. fluviatilis* (Belas *et al*., 1989). This is the first report of a negative effect of increasing salinity values on abundance of *S. lacustris* and *E. mülleri*. *Spongilla lacustris* and *E. mülleri* were more likely to co-exist at sub-sites (Table 3a) suggesting that these species have similar tolerance ranges to salinity, and possible to other parameters. This is supported by previous work (Francis *et al*., 1982; Poirrier, 1974; Belas *et al*., 1989) where lower growth rates at high salinities (0.6 ppt of sodium) were recorded. However, these records were carried out on *E. fluviatilis* in the lab, and in the present study there was no significant abundance differences in this species at high (>0.2 ppt) and low (<0.2 ppt) salinities (Fig. 7). Indeed, it seems unlikely that *E. fluviatilis* abundance is limited by salinity variation within these canals, and it is reported to be the most euryhaline freshwater sponge species (Harrison, 1974) able to tolerate even brackish water levels of 5 ppt (Tendal, 1967). As *E. fluviatilis* was most often segregated at sub-sites (Table 3a) it is possible that this species is able to persist in a different range of conditions to other species here. Glm analyses of *Eu. fragilis* abundance were not driven by salinity, suggesting broad salinity tolerances of *Eu. fragilis* may contribute to its frequent presence at canal sub-sites (Fig. 2) and its cosmopolitan distribution (Pronzato and Manconi, 2001). Using a demarcation value of summer salinity habitat concentration as 0.2 ppt (Fig. 7) could be a convenient indicator of suitability for presence of *S. lacustris* and *E. mülleri*. The value of 0.2 ppt is slightly above the global mean salinity of river water of 0.12 ppt (Thorp and Covich, 2001) but may be useful for lotic habitats and for other holartic species.

Importance of silicon

In addition to salinity, silicon is a key variable impacting distribution of sponge species (Jewell, 1935; Jørgensen, 1944). This effect of silicon is not surprising, as spicules in Spongillidae consist of silicon (SiO_2), and sufficient silicon is an important pre-requisite for sponge growth (Jørgensen, 1944). Indeed, in sponges normal spicule formation ceases at very low silicon concentration 1.2 mgl^{-1} silicon (Garonne *et al*., 1981). According to Jewell (1935) freshwater sponges are limited to waters with silica concentrations >0.5 mgl^{-1}. If these data are appropriate for the four target species studied here, then at some sub-sites (for example BDW012 in Bridgewater canals, Supporting Information 3.1 Table S2) sponges were living below their limits for silicon. However, particle ingestion has been considered as an additional source of silicon (Gaino and Rebora, 2003) to support growth.

Although all four sponge species have siliceous spicules, silicon was a significant factor in *E. fluviatilis* and *Eu. fragilis* only (Table 4a and b; Fig. 5). This apparent importance of silicon was emphasized in the significantly larger size of megasclere spicules in *E. fluviatilis* and *Eu. fragilis* than other UK sponge species (Fig. 3). Both empirical data and experimental studies on sponges have suggested a positive correlation between spicule size and mean silicon level for both freshwater (Jewell, 1935) and marine sponges (Stone, 1970); with controlled studies on freshwater sponge species indicating increased spicule dimensions with higher silicon concentrations (Jørgensen; 1944, 1947; Elvin, 1971). The formation of larger megascleres suggests that an increased requirement for silicon could be a limiting resource for *E. fluviatilis* and *Eu. fragilis*. This idea is supported by early ecological surveys (Stephens, 1920; Jewell, 1935) that also found low silicon levels to be a limiting factor for *Eu. fragilis*. Although low silicon levels have not previously been reported to be limiting for *E. fluviatilis* (Barton and Addis, 1997), *E. fluviatilis* may be unable to persist in habitats with low average summer silicon concentrations. This silicon dependence may, in part, contribute to the separation of *E. fluviatilis* from other *S. lacustris* and *E. mülleri* (Table 3).

Possible silicon dependence for *E. fluviatilis* and *Eu. fragilis* may influence differentiation of spicule-forming cells (sclerocytes), or sclerocyte activity, or both in

these species. In contrast, a broad silicon tolerance range (0 – 20.5 mgl^{-1}) has been suggested for *S. lacustris* (Harrison, 1974). Results here suggest *S. lacustris* has a lower tolerance for silicon than *E. fluviatilis* and *Eu. fragilis*. *Spongilla lacustris* and *E. mülleri* had significantly smaller spicules than both *E. fluviatilis* and *Eu. fragilis* (Fig. 3), suggesting a lower requirement for silicon. Other researchers (Kratz *et al.*, 1991; Colby *et al.*, 1999) found that *S. lacustris* from habitats with low silicon concentration exhibited consistent megasclere length but decreased width than those from habitats with higher concentrations. However, megasclere spicule dimensions in this present study are similar to those of other reference data (Table 2) with no reduction in width values. This suggests a lower tolerance by *S. lacustris* for average summer silicon concentrations found here may exist elsewhere, giving these conclusions a larger interest. Importantly, using a demarcation value of summer silicon habitat concentration as 0.7 mgl^{-1} could be a useful indicator of suitability for growth of *E. fluviatilis* and *Eu. fragilis*, and possibly other cosmopolitan species (e.g. *Eu. carteri*) (Pronzato and Manconi, 2001) (Fig. 8).

Other important factors for sponge abundance

Although ORP is a water parameter accounting for variation in many invertebrate taxa (Williams and Fulthorpe, 2003), this is the first report of a link with ORP and a freshwater sponge species, specifically, *E. fluviatilis*. Values of ORP, together with the pH, have a direct effect on sponges through control of cellular pathways, and an indirect effect by changing microbial activity and possibly, food availability. Larger abundances of *E. fluviatilis* are found in areas with more negative ORP (Table 4b). Negative ORP values with low dissolved oxygen values, form a reducing environment and these values can be associated with dissolved iron pollution, and the formation of ammonium (Dodds, 2002). This tends to negatively impact benthic animals (Cai *et al.*, 2011), and thus it was surprising that only *E. fluviatilis* was affected. *Ephydatia fluviatilis* was often located in isolated stretches of canal (Fig. 2, Table 2b) with low values of dissolved oxygen during summer months (Supporting Information 3.1, Table 2) suggesting this species has a different tolerance range than other UK species. Indeed the frequent presence of *E. fluviatilis* in polluted waters is reported by other researchers (Old, 1932; Harrison, 1974; Økland and Økland, 1996). However, *E. fluviatilis* was the least abundant species

(Fig. 4) and more data points from further studies may help to clarify a relationship with ORP.

Most freshwater sponge species are subjected to seasonal stages of growth, degeneration and gemmulation (Resiwig *et al*., 2009), related, in part, to temporal variation in temperature (Harrison, 1974; Poirrier, 1974; Van de Vyver and Willenz, 1975; Bergquist, 1978). In the present study, maximum and average summer temperatures were important correlates of sponge abundance in *S. lacustris* and *E. mülleri*. It is not clear why all species were not driven by high temperature, as other researchers have also recorded higher abundances of *E. fluviatilis* at higher temperatures (>25 °C) (Harsha *et al*., 1983). Seasonal increases in sponge growth coincide with increased food availability. Sponges feed primarily on plankton (<5 μm) (Reiswig, 1971) which generally increase in abundance as water temperature rises (Fogg, 1986). Alternatively, it is possible that Holartic species (*S. lacustris* and *E. mülleri*) have a different thermal optimum than cosmopolitan species (*E. fluviatilis* and *Eu. fragilis*) and/or cosmopolitan species are less influenced by temporal variations.

Calcium was not important here and was removed from analyses because of collinearity. Previously it has been assumed that calcium, with its effect on regulation of cell metabolism and membrane transport processes, determines the presence of freshwater sponge species (Jewell, 1939; Francis *et al*., 1982). However, it is possible that the remaining UK species, *R. ryderii* may be absent from these habitats as it does not tolerate the calcium range at canal sub-sites (6.32 – 110.30 mgl^{-1}) (Stephens, 1920; Harrison, 1974) and is more often located in habitats with a lower calcium concentration range between 0.5 - 9.0 mgl^{-1} (Ricciardi and Reiswig, 1993). Absence of other sponge species at some sub-sites could be as a result of water parameters or habitat characteristics not recorded in the present study. Habitat characteristics such as degree of canal connectivity (e.g. Cottenie *et al*., 2003), size of isolated canal (e.g. Oertli *et al*., 2002), or age of canal habitat (e.g. Fairchild *et al*., 2000) were not considered here.

Recognising species' habitat requirements could improve understanding of the relationships between freshwater sponges, environmental parameters important to growth, and parameter interactions; as well as being useful for habitat management decisions. Results here suggest that sponge assemblages are influenced by effects working at a regional scale, where water parameters limit the breadth of species distributions. Abundances of *S. lacustris* and *E. mülleri* were higher when salinity values <0.2 mgl^{-1}, and abundances of *E. fluviatilis* and *Eu. fragilis* were higher when silicon concentrations >0.7 mgl^{-1}. The similarity of results from analysis of holartic species with cosmopolitan species adds to the practical value of these relationships, since modelling and field application can be carried out at a level requiring less taxonomic expertise. Water parameters have been used elsewhere (e.g. Rutherford and Kefford, 2005) for assessing and/or protecting aquatic ecosystems. This approach requires identifying important macro-invertebrates and quantifying suitable parameter tolerances as in the present study. Recording parameters instead of identifying species may be less costly and management decisions can be based on comparing water parameters with guideline values (Rutherford and Kefford, 2005).

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Table 1. Variation in water parameter values recorded over one year, including mean and standard deviation (S.D.). Water parameters are ranked according to the percentage of sub-sites where mean values were ≥25% or ≤25% of overall mean value.

Parameter	Range	mean ± S.D.	% of sites ± 25% of mean
Nitrate (mg l^{-1})	1.00 – 37.29	1.47 ± 4.13	100
ORP (mV)	-196.50 – 125.5	16.32 ± 54.69	92
Silicon (mg l^{-1})	0.00 – 6.82	1.79 ± 1.71	83
Salinity (PSU)	0.03 – 0.66	0.18 ± 0.14	83
Phosphate (µg l^{-1})	0.00 – 871.70	86.99 ± 179.90	83
Conductivity (µS cm^{-1})	63.00 – 910.00	282.49 ± 202.81	83
TDS (g l^{-1})	0.06 – 0.85	0.24 ± 0.18	75
Water Velocity ($m.min^{-1}$)	0.00 – 12.00	2.62 ± 5.45	75
Calcium (mg l^{-1})	6.32 – 110.30	39.81 ± 24.29	58
Temperature (°C)	3.72 – 19.35	12.07 ± 4.38	0
DO (mg l^{-1})	3.70 – 14.90	9.70 ± 2.95	0
pH	6.10 – 8.90	7.67 ± 0.68	0

Table 2. Variation in megasclere spicule dimensions of four reported UK freshwater sponge species with mean, standard error (S.E.), standard deviation (S.D.). Length and width of spicules from the present study are compared with those from Ricciardi and Reiswig (R and R) (1993).

Species	Megasclere	$\bar{x}$	S.E.	S.D.	Min		Max	
					Present study	R and R	Present study	R and R
S.lacustris	length	228	5.3	37.3	105	158	278	362
	width	9	0.3	2.2	4	6	13	18
E.mülleri	length	222	3. 8	26.7	158	171	270	311
	width	10	0.4	2.8	4	9	16	20
E.fluviatilis	length	309	5. 9	41.6	244	253	419	439
	width	11	0.3	2.4	5	6	16	19
Eu.fragilis	length	267	5.7	40.1	185	165	360	261
	width	11	0.5	3.6	2	5	21	12

Table 3. Jaccard coefficient measures of mean similarity to analyse co-existence of sponge species (*E. mülleri*, *E. fluviatilis*, *S. lacustris*, and *Eu. fragilis*) at a) all canal sub-sites and b) sub-sites located on isolated stretches of canal.

a)

	Jaccard Measure (*J*)			
	S. lacustris	*Eu. fragilis*	*E. mülleri*	*E. fluviatilis*
S. lacustris	1.00			
Eu. fragilis	0.50	1.00		
E. mülleri	0.77	0.57	1.00	
E. fluviatilis	0.09	0.10	0.07	1.00

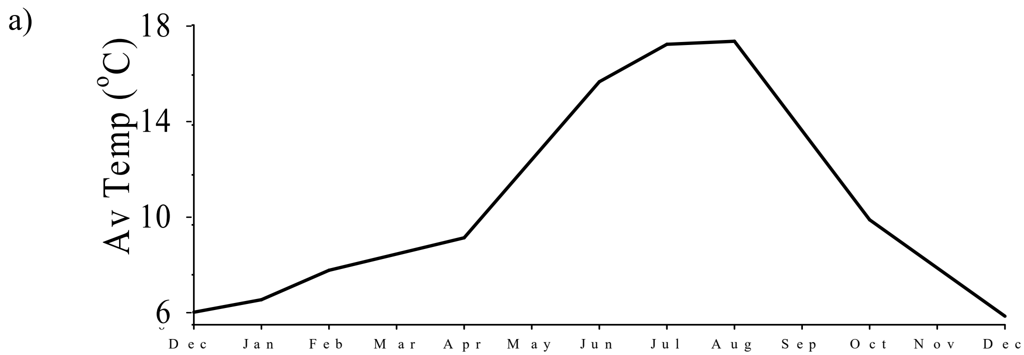
b)

	Jaccard Measure (*J*)			
	S. lacustris	*Eu. fragilis*	*E. mülleri*	*E. fluviatilis*
S. lacustris	1.0			
Eu. fragilis	0.14	1.0		
E. mülleri	0.33	0.33	1.0	
E. fluviatilis	0.25	0.50	0.29	1.0

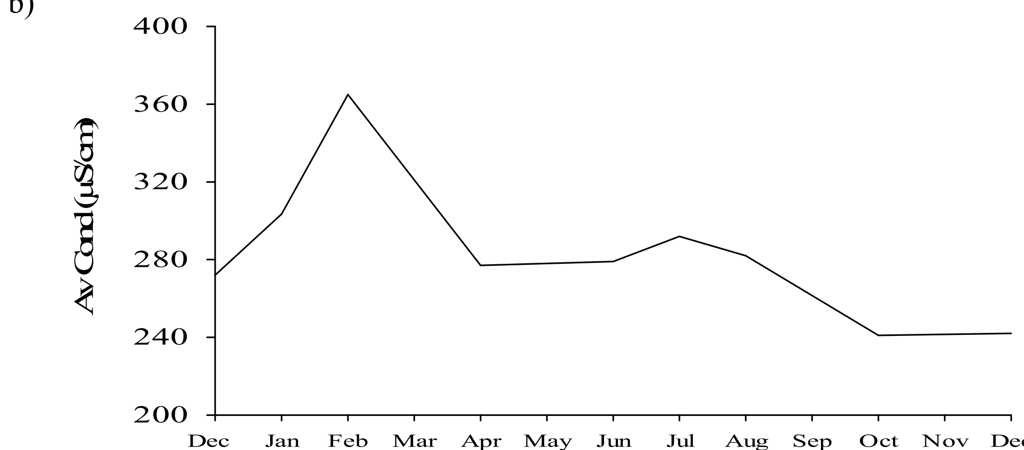
Table 4. Glm analyses for abundance of each sponge species, showing final models and significance levels for average values (Av) or natural log values (*ln*) of water parameters.

		Estimate	S.E.	*t*	*p*
S.lacustris	Intercept	-24.18	12.70	-1.90	
	ln Conductivity	-2.36	0.66	-3.60	< 0.01**
	Maximum Temp	2.17	0.71	3.05	< 0.01**
E. mülleri	Intercept	-0.64	4.53	-0.14	
	ln Conductivity	-1.46	0.52	-2.78	< 0.05*
	Av Summer Temp	0.59	0.26	2.27	< 0.05*
Eu. fragilis	Intercept	0.55	0.72	0.77	
	ln Si	2.37	0.86	2.77	< 0.05*
E.fluviatilis	Intercept	6.85	2.61	2.63	
	ln Si	1.47	0.46	3.18	< 0.01**
	ln ORP	-1.40	0.47	-2.98	< 0.01**

a)



b)



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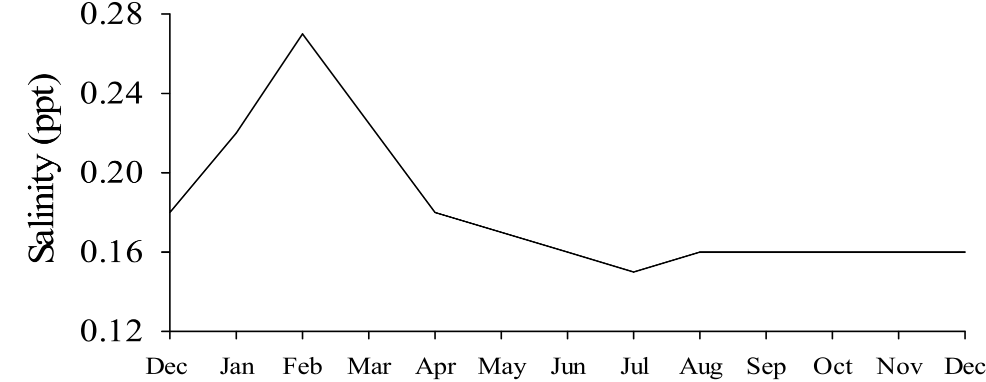

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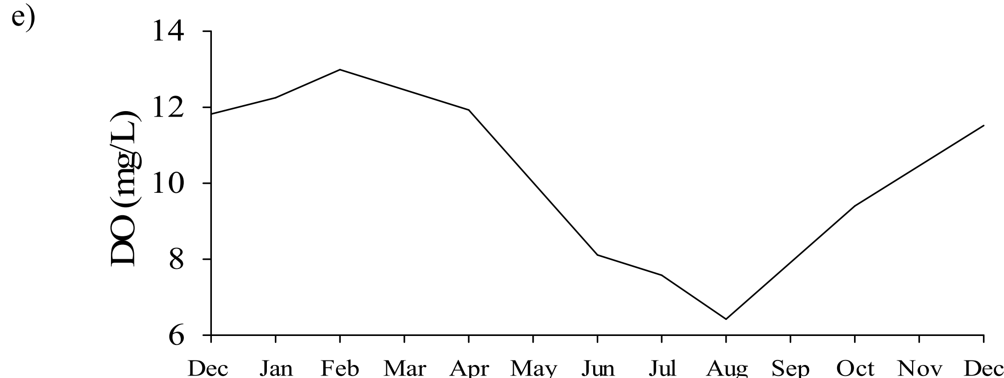

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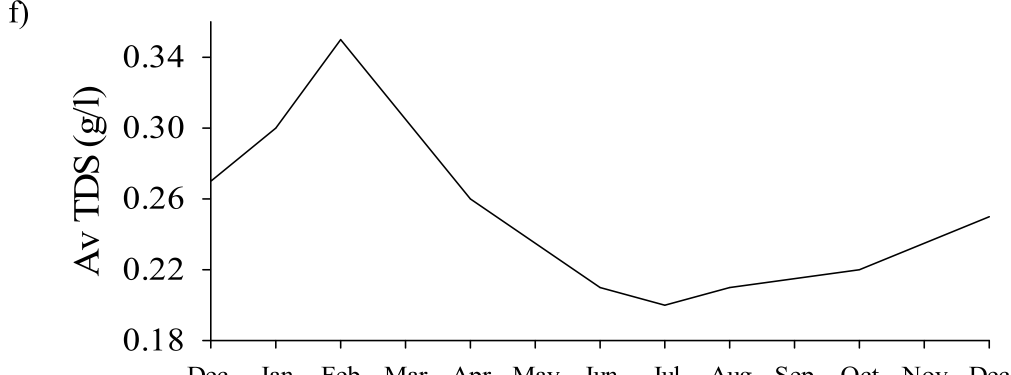

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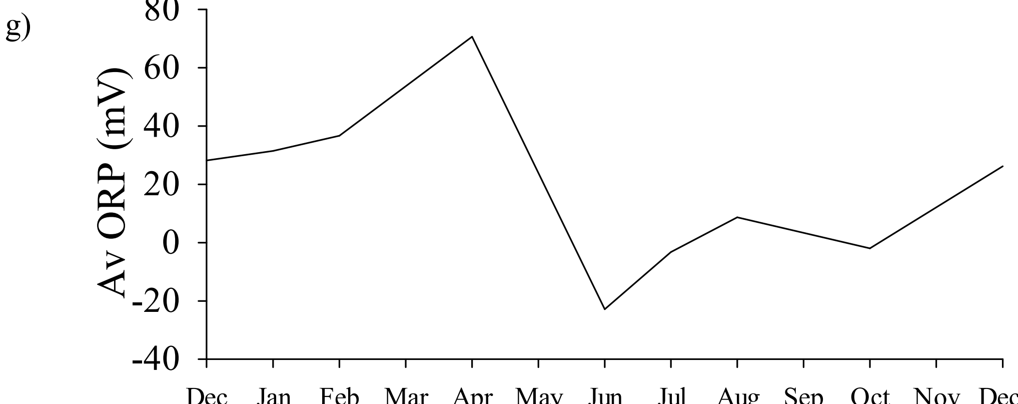

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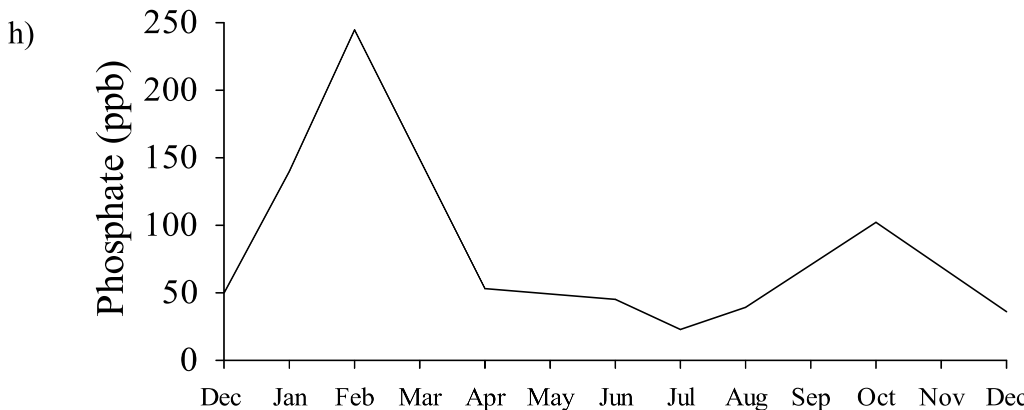

i)

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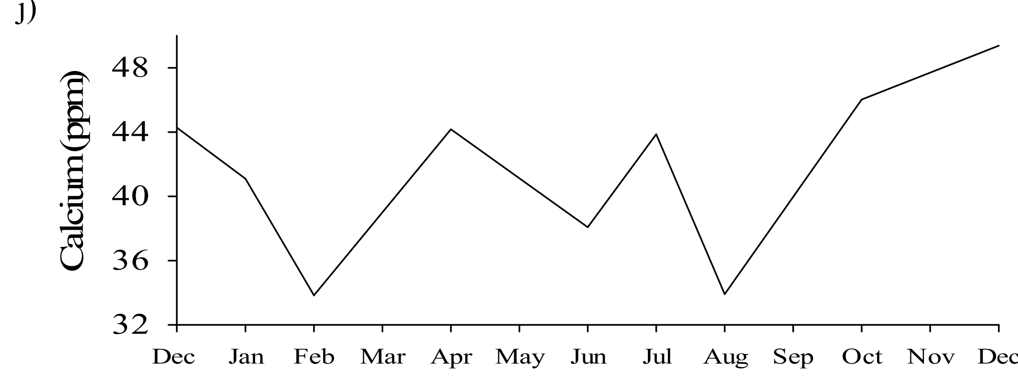

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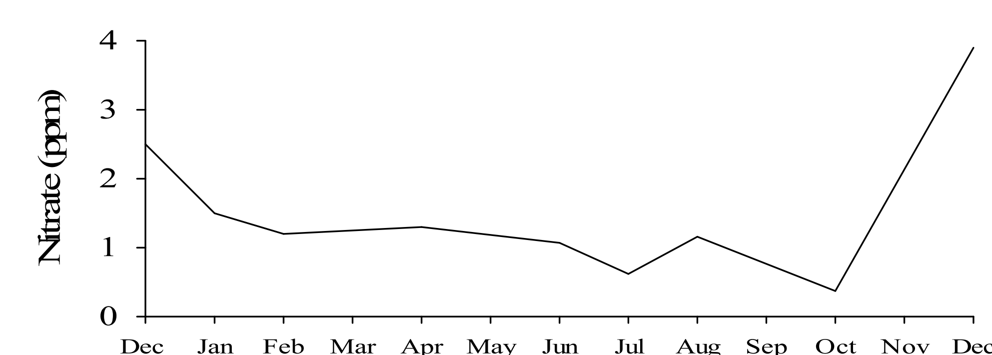

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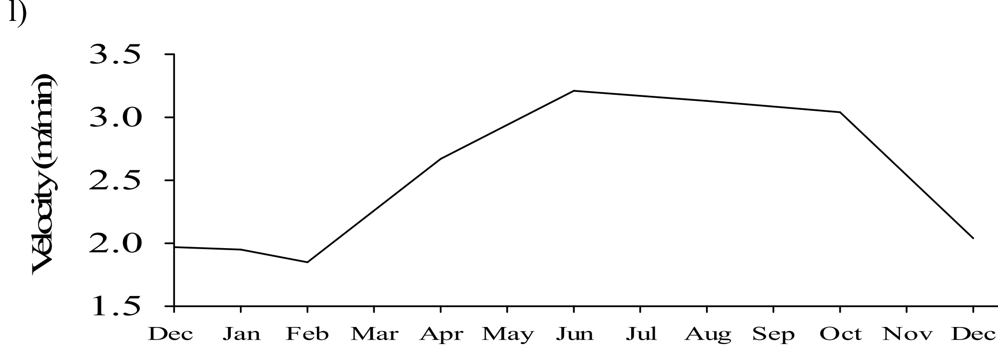


Fig. 1. Changes in average values of water parameters at sampling sites during 13 month sampling period for a) temperature, b) conductivity, c) salinity, d) pH, e) dissolved oxygen, f) total dissolved solids (TDS), g)oxidation reduction potential, h) phosphate, i) silicon, j) calcium, k) nitrate, and l) water velocity taken at 0.4m depth.

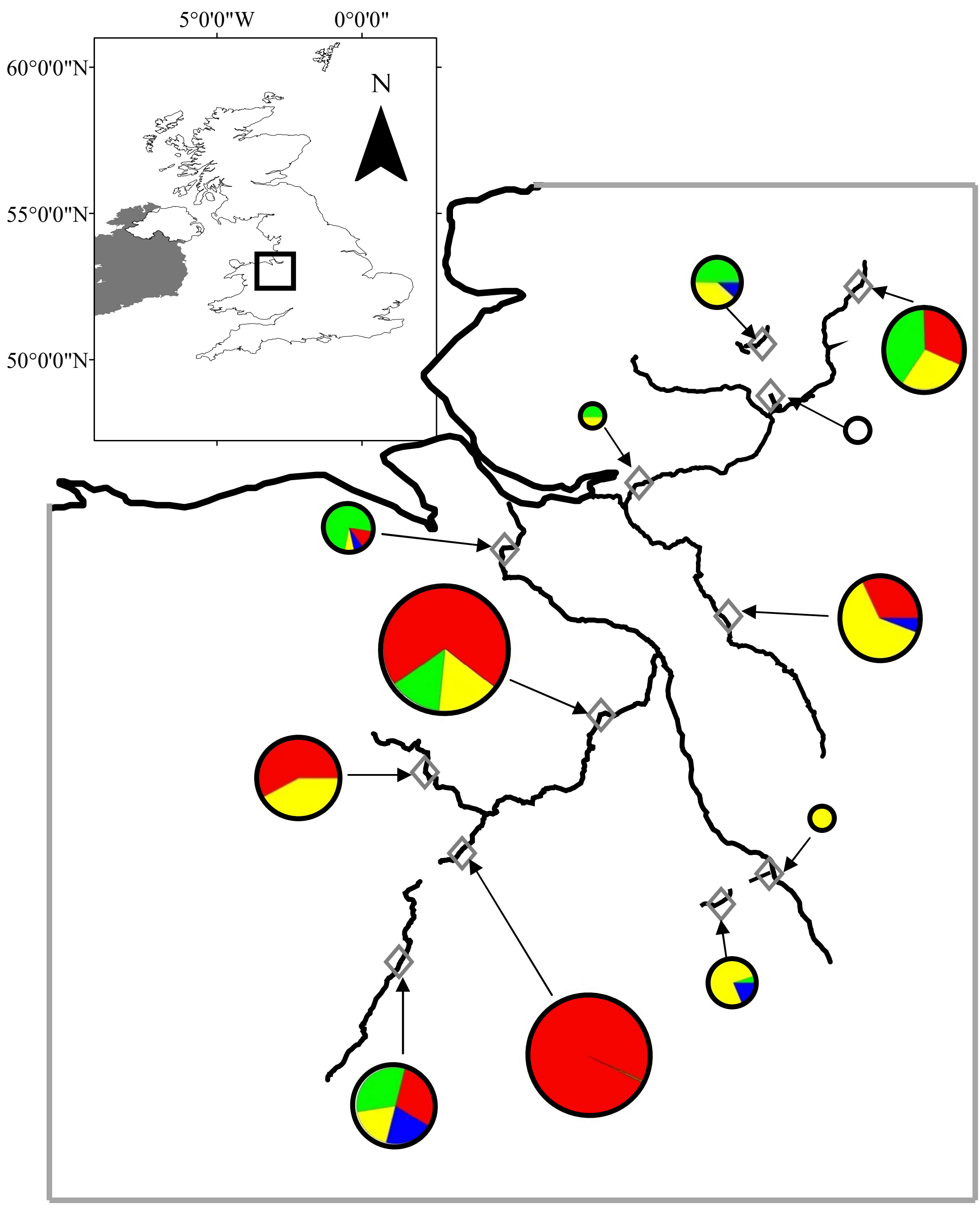


Fig. 2. Approximate location of UK canal sites with species composition (%) and average abundance illustrated by pie charts. Scale: 1cm = 10 km. Only study canals and isolated sections with water are shown. Figure based on: red = *S. lacustris*, green = *E. mülleri*, yellow = *Eu. fragilis*, blue = *E. fluviatilis*, white = no sponges. Area of substrate occupied by sponges: ○ = 0 – 10 cm^2,

○ = 11 - 100 cm^2, ○ = 101 – 1000 cm^2, ○ = 1001- 4000 cm^2.

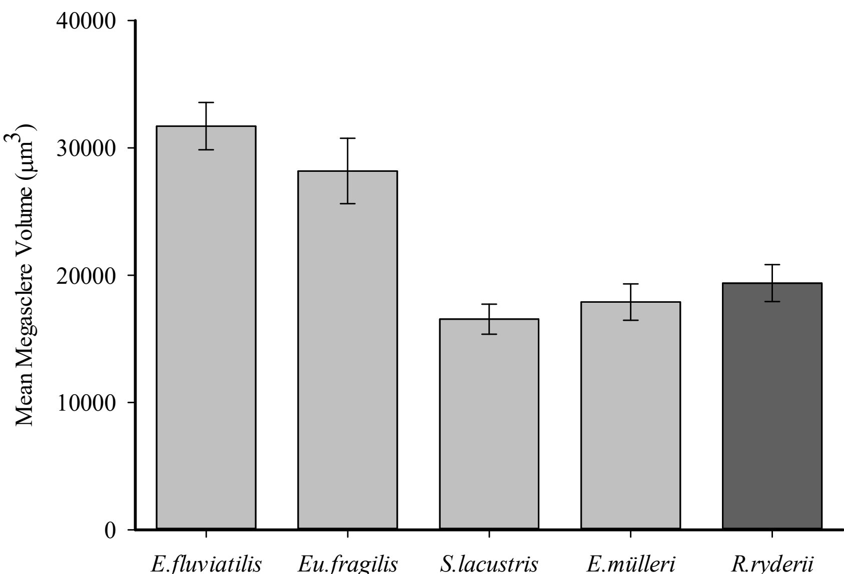


Fig. 3. Variation in megasclere volume (n=50) from UK freshwater sponge species showing a significant difference in mean megasclere size ($F_{4,245}$ =14.41, p = 0.00). Error bars indicate ± 1 S.E. (Although not recorded in the present study, data from *R. ryderii* (Ch 2) are included here for comparison.)

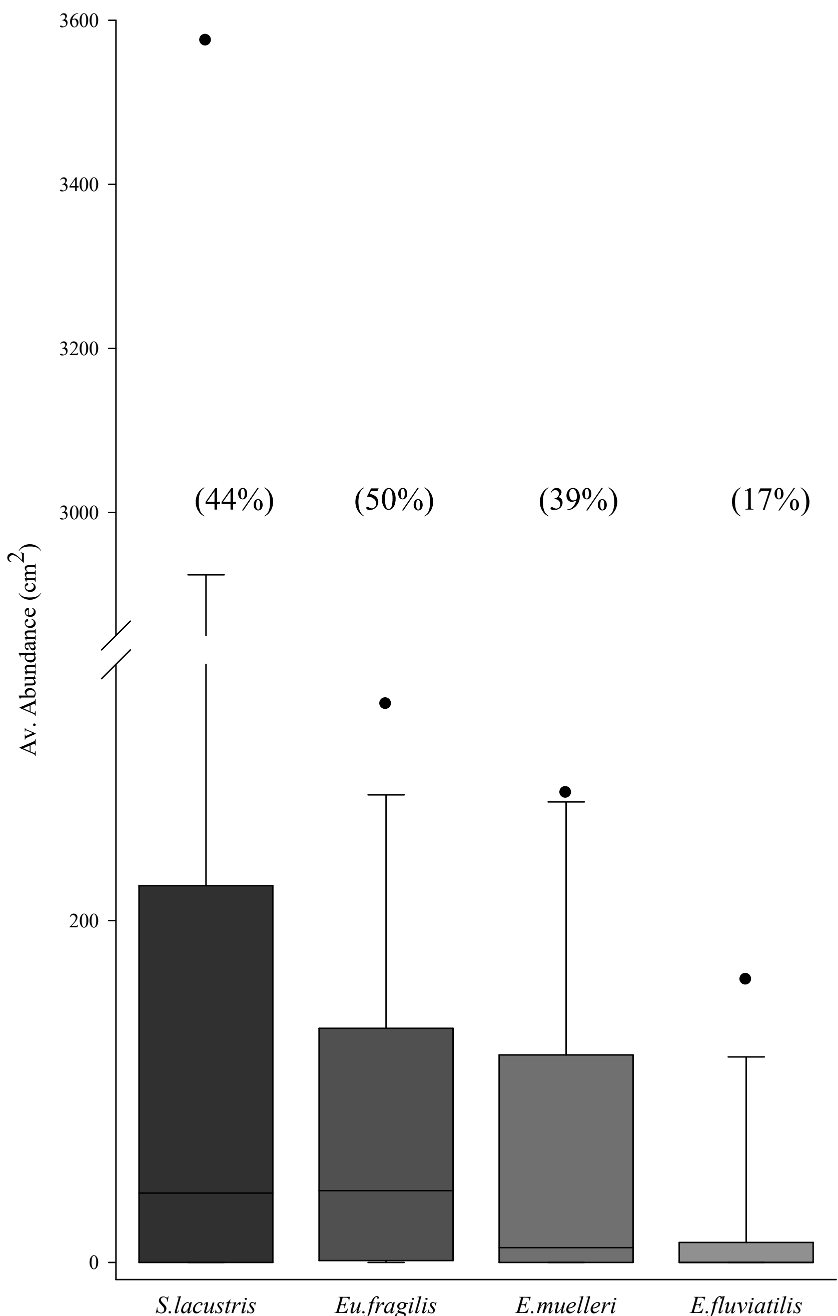
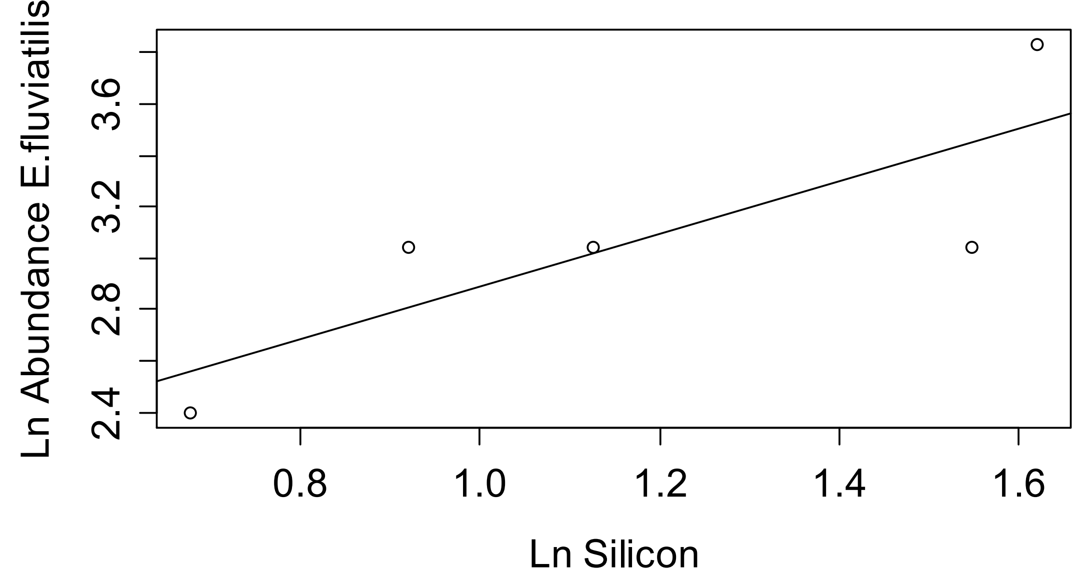


Fig. 4. Variation in abundance of freshwater species at canal sub-sites. Median values are included; the rectangles contain values between the first and third quartiles; the bars connect the extreme values; outlier values are indicated. Percentage occurrence at sub-sites shown in brackets.

a)



b)

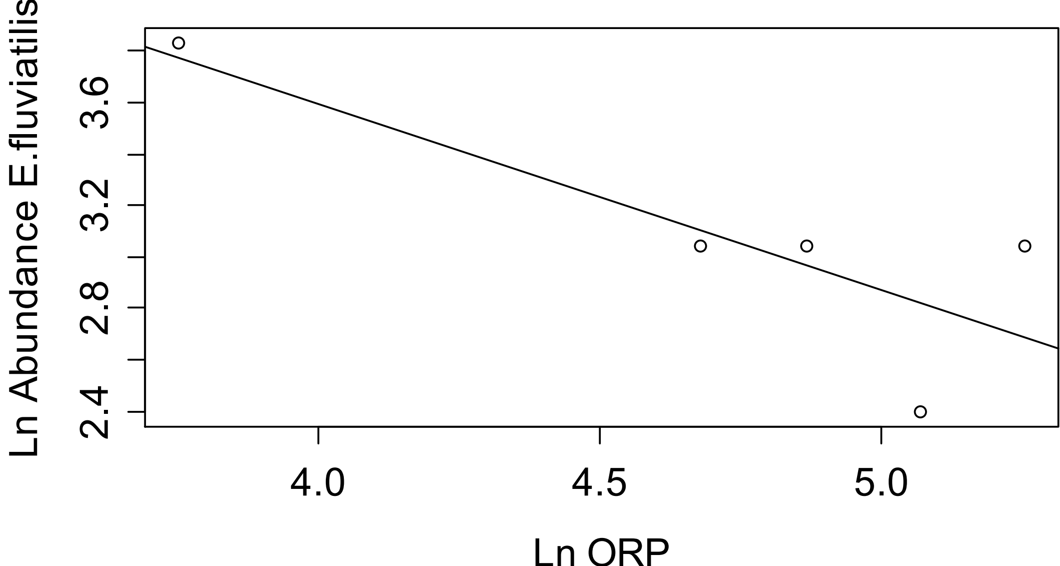


Fig. 5. Correlation of abundance of *E. fluviatilis* at sub-sites with a) variations in natural log (*ln*) of silicon concentration (R^2 = 0.56) and b) variations in natural log (*ln*) ORP values (R^2 = 0.59) based on glm results (zero values removed).

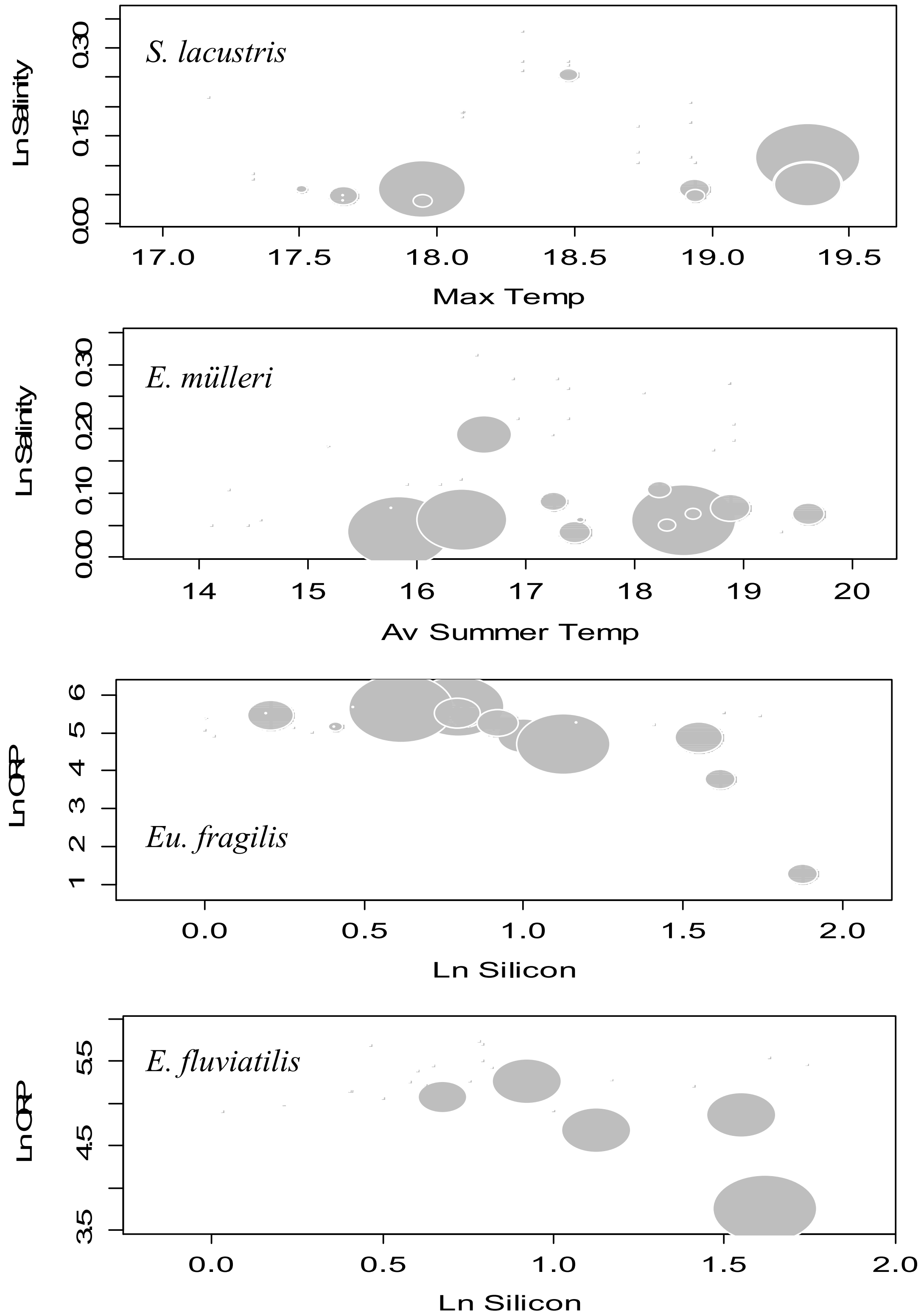


Fig. 6. Bubble plots illustrating changes in population size of freshwater sponges with changes in natural log (*ln*) values of two parameters with substantial support based on glm results (outliers removed).

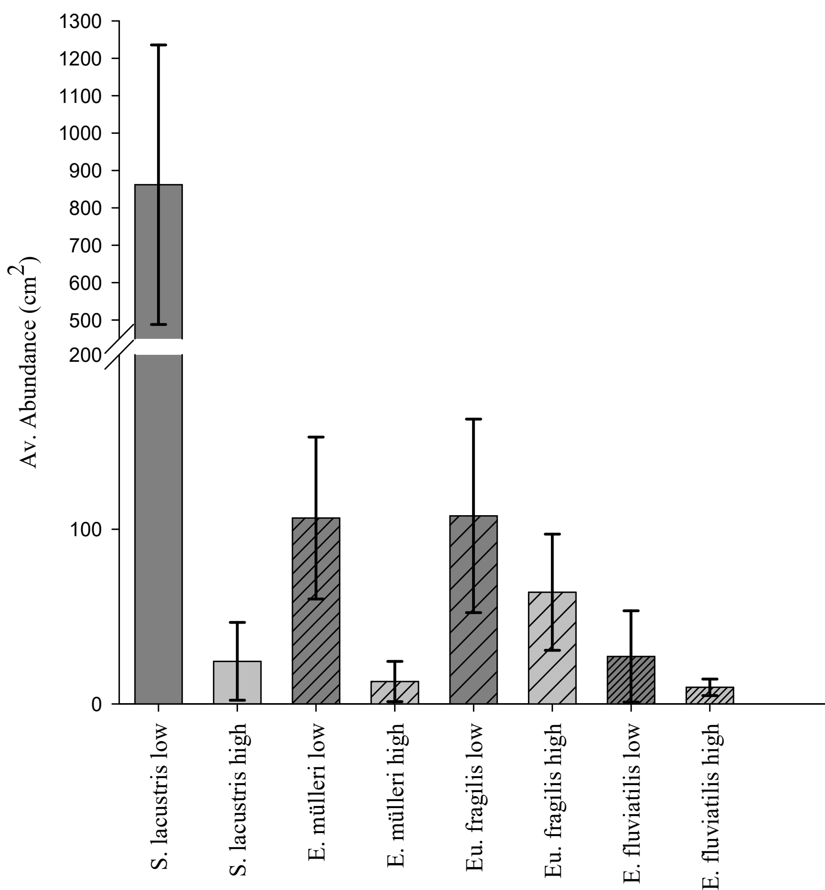


Fig. 7. Sponge species abundance at low (<0.2ppt) and high (>0.2ppt) salinity showing significant difference in abundance of *S. lacustris* (z = 2.36, p <0.05) and *E. mülleri* (z = 2.08, p <0.05). Error bars indicate ±1 standard error.

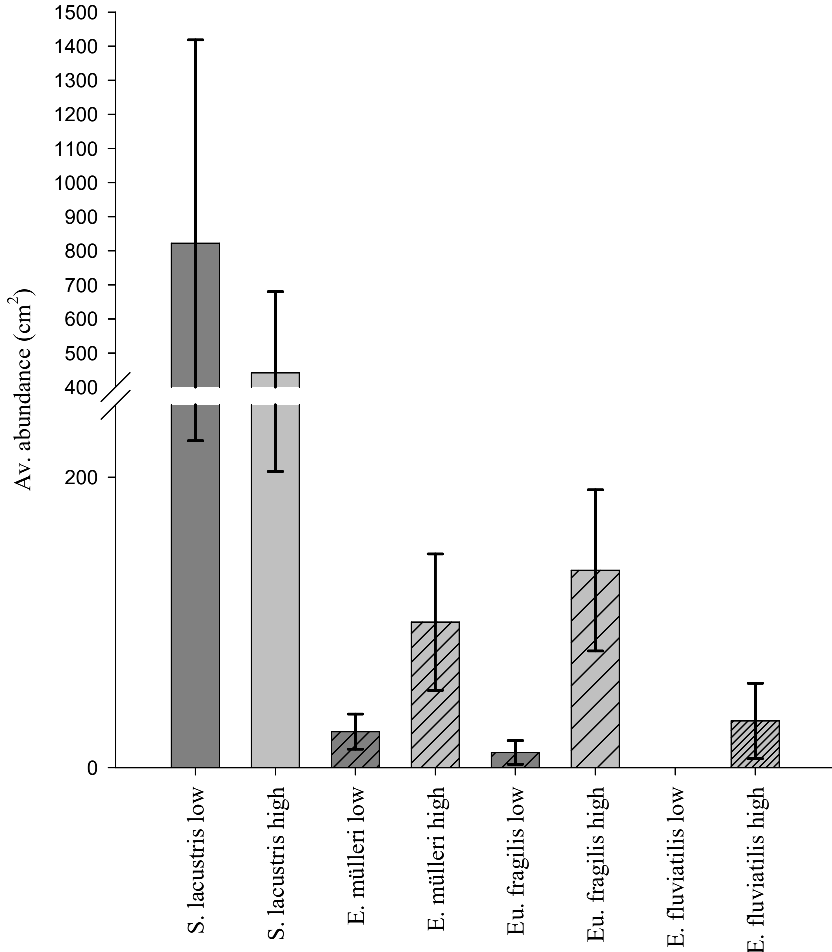


Fig. 8. Average sponge species abundance at low (<0.7 mgL^{-1}) and high (>0.7 mgL^{-1}) silicon concentrations showing a significant difference in abundance of *Eu. fragilis* (z = 3.05, p <0.01) and *E. fluviatilis* (z = 2.25, p <0.05) at high silicon concentrations. Error bars indicate ±1 standard error.

Supporting Information 3.1.

a)

0 5 10 15 20 25

RCD084
RCD092
RCD076
RCD089
RCD072
RCD078
RCD077

b)

0 5 10 15 20 25

SUS088
SUS078
SUS076
SUS072
SUS038
SUS065
SUS059

c)

0 5 10 15 20 25

BDW017
BDW039
BDW036
BDW008
BDW012
BDW035
BDW015

d)

0 5 10 15 20 25

TME183
TME192
TME200
TME154
TME193
TME165
TME210
TME173

e)

0 5 10 15 20 25

MGN082
MGN081
MGN080
MGN071
MGN079

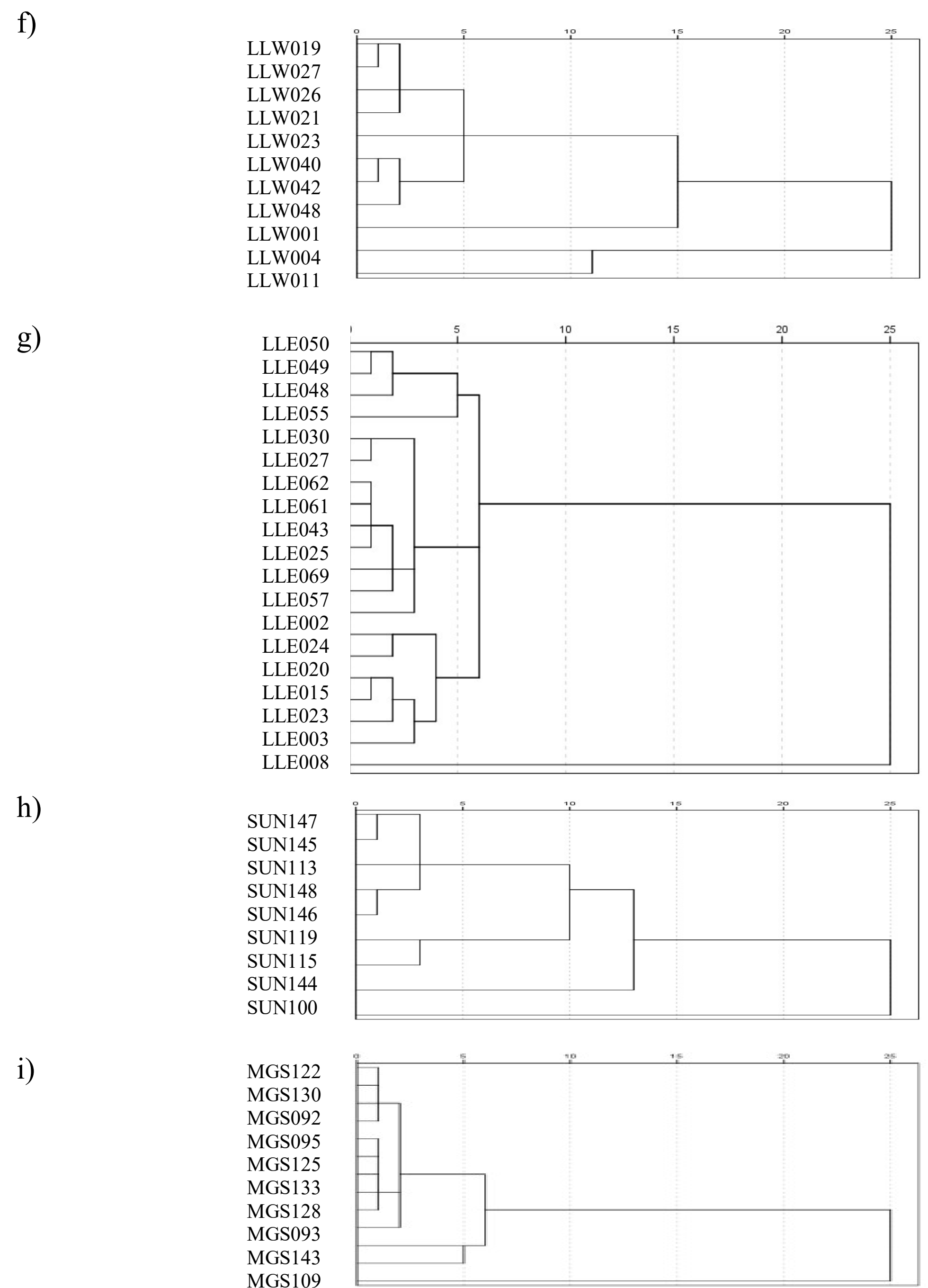


Fig. S1. Clustering pattern of sampling sites (shown as site code) using Euclidean distance, single linkage and Nearest Neighbour method; a) Rochdale Canal sites, b) Shropshire Union Canal south sites, c) Trent and Mersey Canal, d) Bridgewater Canal, e) Montgomery Canal connected sites, f) Llangollen Canal west sites, g) Llangollen Canal east sites, h) Shropshire Union Canal north sites, i) Montgomery Canal isolated sites.

	Temp	Cond	TDS	Sal	DO	pH	ORP	N	P	Si	Ca	Speed
Temp	**1.00**											
Cond	**0.81**	**1.00**										
TDS	**0.72**	**0.98**	**1.00**									
Sal	**0.74**	**0.99**	**1.00**	**1.00**								
Oxygen	0.30	**0.60**	**0.69**	**0.67**	**1.00**							
pH	**0.75**	**0.84**	**0.86**	**0.86**	**0.85**	**1.00**						
ORP	**0.69**	**0.72**	**0.74**	**0.74**	**0.87**	**0.97**	**1.00**					
N	**0.67**	**0.96**	**0.97**	**0.97**	**0.53**	**0.71**	**0.56**	**1.00**				
P	**0.73**	**0.97**	**0.98**	**0.98**	**0.58**	**0.76**	**0.60**	**0.99**	**1.00**			
Si	**0.77**	**0.96**	**0.98**	**0.99**	**0.66**	**0.85**	**0.73**	**0.95**	**0.97**	**1.00**		
Ca	**0.76**	**0.97**	**0.98**	**0.98**	**0.75**	**0.93**	**0.82**	**0.91**	**0.94**	**0.97**	**1.00**	
Speed	**0.76**	**0.98**	**0.99**	**1.00**	**0.65**	**0.86**	**0.73**	**0.96**	**0.98**	**0.99**	**0.98**	**1.00**

Fig. S2. Relationships between water parameters recorded at sites. R^2 values indicating the proportion of the variance explained by the linear relationship. Values of R^2 >0.5 shown in bold type.

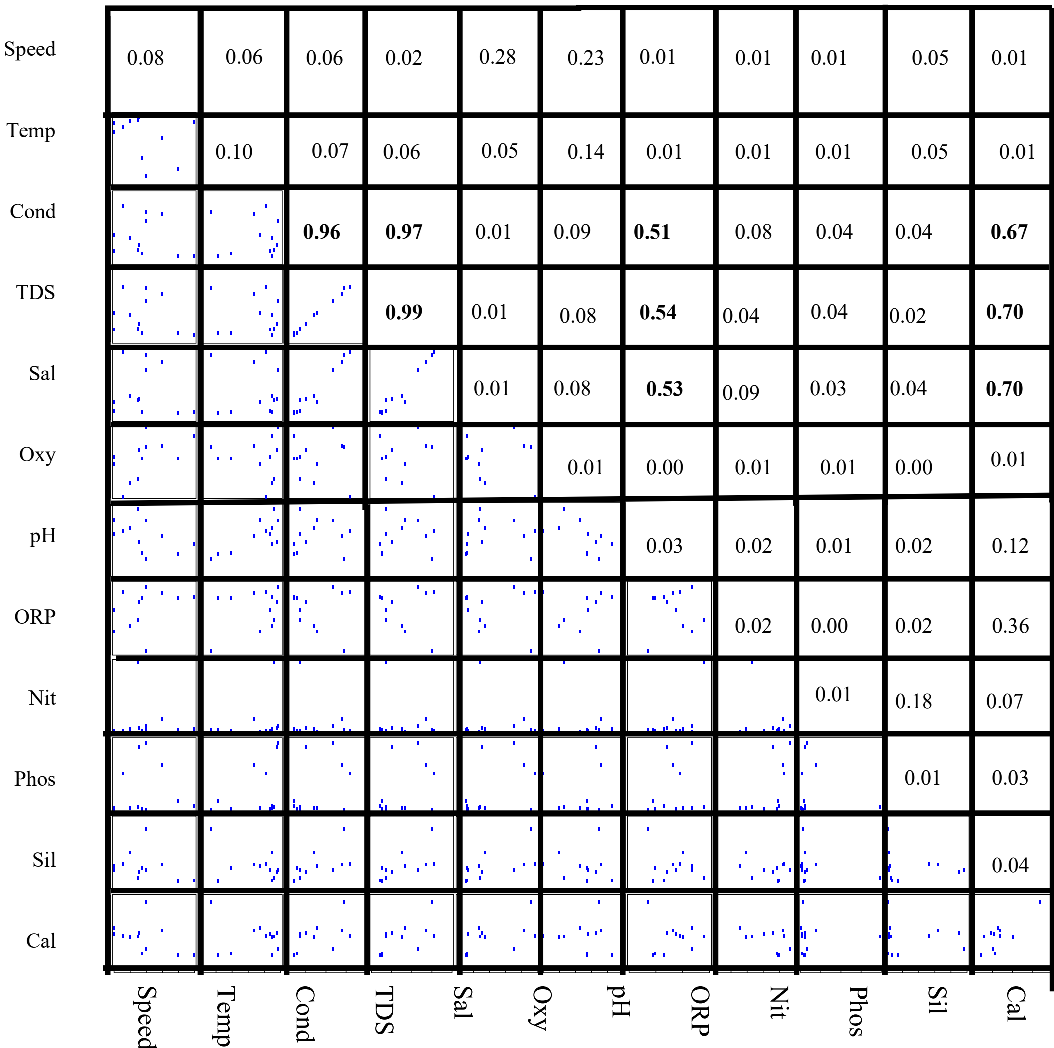


Fig. S3. Relationships between water parameters recorded at sub-sites. Plots of correlation shown in lower left. R values indicating the proportion of the variance explained by the linear relationship shown in upper right. Values of R >0.5 shown in bold type.

Table S1. Significance of water parameter variables recorded in the present study.

Variables recorded at 0.4m	Expected range	Year round range	Summer range	General significance to sponges - from Harrison (1974) unless indicated.	Significance to UK sponge species - from Harrison (1974) unless indicated
Mean water temperature (°C)	2 - 20	3.72 - 19.6	14.12 – 19.6	May influences sponge life cycle, morphological features or growth.	Low temperatures inhibit gemmule development in *S. lacustris*. *E. mülleri* is less tolerant than *S. lacustris* of lower temperatures (Økland and Økland, 1996).
Mean Conductivity (µS cm^{-1})	60 - 1200	19 - 910	19 - 674	Index of dissolved inorganic matter. Increases in conductivity from allochthonous material following rain (Melão and Rocha, 1999). A limiting factor at extremes for some sponge species. Low levels inhibit spicule formation.	*S. lacustris* and *Eunapius fragilis* occurs more frequently at areas with low conductivity <100 µS/cm (Paduano and Fell, 1997; Økland and Økland, 1996).
Mean water salinity (ppt)	0.03 - 0.6	0.03 - 0.66	0.04 – 0.39	Freshwater has salinity <0.5ppt, brackish water has salinity of 0.5 – 30ppt. Euryhaline species may be able to out-compete other species in stretches of canals near to the port with higher salinity values (chapter2).	High levels (>0.6ppt) have negative impact on growth rate of *E. fluviatilis* (Belas *et al.*, 1989). The highest level recorded value for *E. fluviatilis* is 5.00 ppt (Tendal, 1967).
Mean pH	6 - 10	5.09 - 9.00	5.09 - 9.00	Low values of pH inhibit gemmule hatching. High values inhibit skeletal spicule formation in some species.	*Eunapius fragilis* and *S. lacustris* tolerant to wide range of pH. However, *S. lacustris* occurs more frequently at areas with low pH (Paduano and Fell, 1997; Økland and Økland, 1996).
Mean dissolved oxygen content (mg l^{-1})	4 – 14.5	4.0 - 15.4	4.0 – 12.71	Oxygen is required for aerobic respiration. Oxygen levels may drop in areas with pollution or of canals with slow moving water.	*Eunapius fragilis* and *E. mülleri* tolerant of low oxygen levels.
Mean value of total dissolved solids (TDS) (g l^{-1})	0.1 - 0.9	0.049 - 0.545	0.049 – 0.514	Increases in TDS from allochthonous material following rain (Melão and Rocha, 1999). Particles of dissolved solids may clog filtering system of sponge especially when growing on vertical canal substrate rather than under rocks.	*Eunapius fragilis* is tolerant to higher values of TDS than other species. High levels of TDS may prevent sponge growth from gemmules.

Oxidation Reduction Potential (ORP) (mV)	-30 - +100	-196 - 125.5	-196 - 110	Measures presence of reducing substances and a water system's capacity to either release or gain electrons in chemical reactions. Together with pH it controls cellular processes (Williams and Fulthorpe, 2003).	No specific data for sponges, but it has been suggested to be an important variable when assessing freshwater invertebrate communities (Williams and Fulthorpe, 2003).
Mean phosphate concentration (P-PO_4) (ppb)	10 - 800	0 - 871.7	0 - 452.8	Phosphate levels may increase with agricultural run-off. Phosphate influences distributional patterns in some sponge species.	*Ephydatia* species are more tolerant of eutrophication than *S. lacustris* (Økland and Økland, 1996).
Mean silicate concentration (SiO_2) (ppm)	0.1 - 5	0 - 6.82	0 – 5.55	Silicate concentration in canal water determined by water runoff in drainage basin and mixing of water from several sources. Sponges transfer Si from the water to the sediment. Silicate levels determine degree of skeletal spicule development.	A level above 0.5 ppm needed for sponge growth (Jewell, 1935). Growth of *Eunapius fragilis* and *E. mülleri* limited by silicate values <0.7ppm.
Mean calcium concentration (Ca^{2+}) (ppm)	5 - 200	6.32 - 110.3	8.67 – 98.2	Calcium regulates some aspects of cell metabolism through control of membrane transport processes. Calcium concentration in canal water determined by water runoff in areas with limestone and mixing of water from several sources. Jewell (1939) reported that calcium was the most important factor determining the distribution patterns of sponges. Levels below 18ppm can limit growth in some sponge species.	*E. fluviatilis* unable to survive at levels above 150 (Francis *et al.*, 1982). Little or no growth of *E. fluviatilis* at levels below 15 ppm (Belas *et al.*, 1989). Growth of *S. lacustris* not influenced by calcium and this species and *Eunapius fragilis* have been shown to occur more frequently at areas with low calcium (Paduano and Fell, 1997; Økland and Økland, 1996).
Mean nitrate conc (N-NO_3) (ppm).	1 - 50	0 - 37.29	0.09 – 4.50	Nitrate levels may increase with agricultural run-off.	Nitrate influences distributional patterns in some sponge species
Surface water velocity ($m.min^{-1}$)	0 - 10	0 - 12	0 - 12	Oxygen levels and TDS values may be affected by slow moving water. Mild currents transport nutrients High water velocity may prevent gemmule settlement.	Suspension-feeding freshwater invertebrates are strongly influenced by water velocity (Thorp and Covich, 2001).

Table S2. Canal sites including sub-sites with high average salinity (>0.2 mgl^{-1}), high silicon values (>0.7 mgl^{-1}), low dissolved oxygen (<80 mgl^{-1}), high water velocity (>4 $m.min^{-1}$), and negative yearly values of ORP. For each site, the number of sub-sites removed as outliers during glm analyses of sponge species' abundance are indicated.

Canal	Isol-ated canal	High av salinity	High av silicon	Low summ er DO	High av velocity	– ve ORP	Sub-sites removed in glm analyses
SUC - North	n	y	y	n	n	n	0
SUC - South	n	y	y	n	y	n	0
Llangollen - east	n	n	y	n	y	n	1 (abundance of *Eu. fragilis*)
Llangollen - west	n	n	y	n	y	n	1 (abundance of *E. mülleri*)
Montgom-ery- north	n	n	y	y	n	n	0
Montgom-ery- south	y	n	y	y	n	n	1 (abundance of *E. mülleri*). 1 (abundance of *E. fluviatilis*)
Newport	y	y	y	y	n	y	1 (abundance of *E. fluviatilis*)
Bridge-water	n	n	n	n	n	y	0
Rochdale	n	n	y	n	n	n	0
Trent & Mersey	n	y	y	n	n	n	0
Manchester Bury & Bolton	n	n	y	n	n	n	0
Manchester Bury & Bolton	y	n	y	y	n	n	0

Table S3. Using the best glm for abundance of each sponge species, statistically significant parameters are indicated for models with substantial support,

	Inter-cept	*ln* Cond	Max or Summ T	Max DO	Spr DO	Aut or Win DO	*ln* Si	*ln* ORP	*ln* P	df	AIC	Δ AIC
S. lacustris	-24.19	-2.36	2.17							28	139.8	0
	-9.31			1.78	-1.02					28	140.7	0.86
	-62.54		2.5	1.47						28	141.2	1.4
	-9.29			1.43		-0.81				28	141.7	1.92
E. mülleri	-0.64	0.59	0.59							26	116.1	0
Eu. fragilis	0.55						2.37			27	121.9	0
	-3.47						3.20	0.68		27	122.4	0.57
	-3.04					0.29	2.45			27	122.7	0.79
	-0.07						2.47		0.22	27	122.9	1.04
E. fluviatilis	6.85						1.47	-1.40		26	69.5	0
	-4.03	0.64					1.86			26	70.7	1.15

Table S4. Post hoc test with multiple comparisons of species megasclere volume showing mean significant difference at 5% level as significant (sig) or not significant (ns).

Species		Mean difference	Significance
S.lacustris	*E.mülleri*	-1345.67	ns
	E.fluviatilis	-15170.63	**sig**
	Eu. fragilis	-11648.93	**sig**
E.mülleri	*S.lacustris*	1345.67	ns
	E.fluviatilis	-13824.96	**sig**
	Eu. fragilis	-10303.26	**sig**
E.fluviatilis	*S.lacustris*	15170.63	**sig**
	E.mülleri	13824.96	**sig**
	Eu. fragilis	3521.70	ns
Eu. fragilis	*S.lacustris*	11648.93	**sig**
	E.mülleri	10303.26	**sig**
	E.fluviatilis	-3521.70	ns

Chapter 4. Identifying freshwater sponges using DNA barcoding and morphology, and comparison with marine sequence data.

Summary

Freshwater sponges are commonly found in many UK habitats but they are difficult to identify, leaving biodiversity estimates are incomplete. Partitions of the cytochrome oxidase subunit 1 (CO1) gene are frequently used as the standard marker for DNA barcoding in animals. An analysis of a partial sequence (553 bp) of COI gene sequences was conducted to determine variation within and between freshwater sponge species, and was followed with an analysis of marine species, and assessed if the COI locus can be used for DNA barcoding in sponges. The resolution power of the COI gene was complimented with a more variable ITS rDNA sequence data from UK freshwater sponge species. Alternatively, spicule and gemmule morphology has been used for species identification in freshwater taxa. However, with species' delineation depends on sufficient character separation, and the interpretation of sometimes variable traits. As there has been no taxonomic study of UK freshwater sponge species, the present study expands on barcoding effort by incorporating these data with ITS sequence data, and with morphological data. Low variability of COI gene sequences was found, with low nucleotide diversity. This is in agreement with reports on cnidarians and points to a high conservation of mtDNA in diploblastic phyla. An integrated approach using genetic and morphological characteristics to identify sponge species identification was suggested.

Introduction

Reliable and accessible species' identification methods are fundamental to research in biodiversity, and evolutionary biology (Monaghan *et al.*, 2005; Hooper *et al.*, 2005). There is an increasing need to accelerate the pace of species' identification because of the constant threat of biodiversity loss (Monaghan *et al.*, 2005). However, estimates suggest that only 10% of the animal species have been identified (Packer *et al.*, 2009), with the remaining species belonging to "taxonomically difficult" groups (Chivian and Bernstein 2008). Thus, evaluating species identification methods is important to assist in overcoming knowledge gaps in biodiversity especially in understudied taxa.

With over 8,000 described species, and an estimated 7,000 un-described species (Hooper and van Soest, 2002), the phylum Porifera represents a prime example of an understudied group with taxonomic difficulties (Wörheide and Erpenbeck, 2007). Although most members of Porifera are marine, there are about 250 species of freshwater sponges (Order: Spongillida) (Erpenbeck *et al*., 2011; Hooper and van Soest, 2002; Manconi and Pronzato, 2002) that inhabit a wide range of freshwater habitats, including lakes, rivers (Penney and Racek, 1968; Reiswig *et al*., 2009) and canals (Chapter 2). Freshwater sponges are divided into six extant families: Spongillidae, Potamolepidae, Lubomirskiidae, Malawispongiidae, Metschnikowiidae, Metaniidae (Manconi and Pronzato, 2002). Spongillidae consist of the largest number of known species (Manconi and Pronzato, 2002). In fact, all six reported UK species (see Chapter 2) of freshwater sponge are members of the Spongillidae: *Spongilla lacustris* (Linnaeus, 1759), *Ephydatia fluviatilis* (Linnaeus, 1759), *Ephydatia mülleri* (Lieberkühn, 1855), *Eunapius fragilis* (Leidy, 1851), *Racekiela ryderii* (Potts, 1882), and *Trochospongilla horrida* (Weltner, 1893). However, even differentiating between these six species can be difficult (Wörheide and Erpenbeck, 2007).

Rather few characteristics can be applied to differentiate between species of Porifera; for example, gross external morphology can rarely be used because of the highly variable nature of a sponge's external appearance (Reiswig *et al*., 2009, Harcet *et al*., 2010). Sponge taxonomy, therefore, typically relies upon the presence and shape of microscopic skeletal spicule elements: megascleres, microscleres (in some species) and especially gemmuloscleres associated with gemmules (Reiswig *et al*., 2009) (see Chapter1). However, species' identification problems arise when spicules show interspecific similarities. For example, the size and shape of megasclere of *Eunapius fragilis* are similar to those *Ephydatia fluviatilis* (Chapter 2). Also, as gemmules are not present in Lubomirskiidae, Malawispongiidae, or Metschnikowiidae (Manconi and Pronzato, 2002), critical gemmule morphological traits cannot be used in identification of species from these families. Species' delineation depends on sufficient character separation, the thoroughness of study, and the interpretation of sometimes variable traits (Monaghan *et al*., 2005).

Where morphological characters provide insufficient differentiation for species determination, molecular data have become widely used to define species (Hebert *et al*., 2004b; see Chapter 1). DNA barcoding, the use of a single, standardised genetic locus to delineate species across different taxa was suggested by Hebert *et al*. (2003a, b). The typical DNA barcoding locus is based on a mitochondrial cytochrome c oxidase subunit 1 (COI) gene sequence, and relies on the feature that, for many species, the level of interspecific variation at COI is greater than intraspecific variation. This results in a distinct gap in the distribution of sequence variation, or the 'barcoding gap', that allows species to be identified (Hebert *et al*., 2003a, b; Shearer and Coffroth, 2008) (Supporting Information 4.1, Fig. S1). While there is an apparent 'barcoding gap' of typically 2–3% sequence divergence (Hebert *et al*., 2003b) for some invertebrate taxa (e.g. moths, Hebert *et al*., 2004b; spiders, Barrett and Hebert, 2005; butterflies, Hajibabaei *et al*., 2006), a low mean COI sequence divergence (1% or less) has been observed in other groups (e.g. Anthozoa, Shearer and Coffroth, 2008). However, DNA barcoding has not previously been investigated in UK freshwater sponges.

Currently, sponges are the only non-bilaterian invertebrate phylum to be barcoded through a global campaign (Sponge Barcoding Project (SBP), www.spongebarcoding.org) using emerging protocols. The use of DNA barcoding using COI has assisted the classification of marine sponge taxa (e.g. Raleigh *et al*., 2007) and freshwater sponge taxa inhabiting ancient lakes (e.g. Lake Baikal, Schröder *et al*., 2003; Lake Tanganyika, Erpenbeck *et al*., 2011). In addition, SBP protocol suggests the use of internal transcribed spacers (ITS) of rDNA gene sequences to optimise species differentiation. These gene fragments used for barcoding also harbour considerable phylogenetic signal, which have show congruence in other sponge studies (Erpenbeck *et al*., 2011; Itskovich *et al*., 2007; Meixner *et al*., 2007). As there has been no barcoding study of UK freshwater sponge species, the present study expands on SBP barcoding effort by incorporating these data with morphological methods (spicules).

Thus, the present study extends biodiversity research to quantify COI sequence divergence between freshwater sponge species using data from targeted UK sampling sites and augmented with unidentified sponge material from Museum collections. By combining these data with those from other freshwater sponges (see Chapter 1), investigations assessed (1) if freshwater sponge species exhibit a barcoding gap at COI and thus (2) the COI locus can be used for DNA barcoding in this group; (3) phylogenetic relationships using COI and ITS sequences; and (4) if spicule morphology is required for reliable species' identification. Finally, a similar genetic evaluation of intra- and interspecific variation in COI in marine sponge sequences from GenBank was carried out to make a general assessment of the usefulness of DNA barcoding using COI for Porifera as a whole.

Methods

Sponge material – location and data sources

Several sources of sponge specimens were used. First, surveys between 2008 and 2012, for freshwater sponges were performed at 100 sites along 8 canals located in the north-west UK (Fig. 2, Chapter 3). All survey sites were located under a bridge, along the water line, and the canal substrate was examined for presence of sponges. At sites where sponges were present (Supporting Information 4.1, Table S1), three sub-samples, bearing gemmules if possible, were collected from each colony to a water depth of 40 cm. Sub-sampling sponge tissue was important to ensure that samples were from each species. To collect as many species as possible, additional sponge samples were obtained from natural UK freshwater habitats (n=6) and from the Natural History Museum, London (n=8) (Supporting Information 4.1, Fig. S2). Other sponge samples were obtained from researchers elsewhere in Europe (Ireland, Germany) and Australia (n=7). Sponge samples were stored in 100% ethanol.

Taxonomy – Microscopy

Samples were examined under a stereomicroscope to separate sponge tissue from epibiota. Species were identified based on spicule morphology following taxonomic keys of Pronzato and Manconi (2001) and Manconi and Pronzato (2002) (for a simplified version of these keys, see Chapter 2) with additional information on

gemmule foramen structure (see Chapter 1). Samples (~1 cm^3) from the UK survey were boiled in 100% nitric acid for 2 min and then left for ~ 24 h, to separate spicules from the tissue. After three washes in water and re-suspension in 100% ethyl alcohol, spicules were transferred to slides. Spicules were examined at 200 times magnification; images were captured with a digital camera and analysed with an image analysis system. Up to three types of siliceous spicules were analyzed for sponge identification: megascleres, which make up the framework of a sponge skeleton; microscleres, spicules with a smaller size range; and gemmuloscleres, which surround the gemmules. The mean length and width of megascleres from UK sponges were determined from >25 spicules from several specimens (following Pronzato and Manconi, 2001).

Taxonomy – Sequencing

Total genomic DNA was extracted from sponges using a DNeasy tissue kit (DNeasy, Qiagen Inc, Hilden, Germany) following the manufacturer's instructions, except that after incubation in lysis buffer the tubes were centrifuged to separate spicules from the buffer. DNA concentration was quantified using a QuantiT dsDNA assay kit and a Qubit fluorometer (Invitrogen, Carlsbad, CA, USA).

Polymerase chain reaction (PCR) was used to amplify a 553 bp fragment of the 5′ end of the cytochrome c oxidase subunit I mtDNA gene, using LCO1490/HCO2198 primers of Folmer *et al*. (1994) (Supporting Information 4.1, Fig. S3a).

LCO1490: 5' - GGT CAA CAA ATC ATA AAG ATA TTG G - 3'

HCO2198: 5' - :TAA ACT TCA GGG TGA CCA AAA AAT CA - 3'

Subsequent PCR techniques were used to amplify a 620-643bp the ITS2 region situated between the 5.8S and 28S rDNA segments using the ITS3 and Ep2 primers of Addis and Peterson (2005) (Supporting Information 4.1, Fig. S3b).

ITS3: 5'-GTCGATGAAGAACGCAGC-3'

Ep2: 5'–CTYYGACGTGCCTTTCCAGGT-3'

PCRs were set up in a 20 µl final reaction volume that contained ~10 ng genomic DNA containing the following: 10 µl 2X GoTaq Green (Promega, Southampton, UK), 0.4 pmol of each primer (Eurofins MWG Operon, Germany), 2.0 µl 10X BSA

(New England Biolabs Inc., Hitchen, UK). Thermal cycling conditions were: 94 °C for 4 min, followed by 35 cycles at 94°C for 45 s, 45°C for 30 s, 72°C for 45 s, and a final 72°C for 8 min. PCR products were then purified with 0.15 µl shrimp alkaline phosphatase (1000U ml^{-1}) (USB, UK) and 0.03 µl Exonuclease I (20000Uml^{-1}) (New England BioLabs, UK) following the manufacturer's protocol. Sequencing was performed using BigDye v.3.1 chemistry (Applied Biosystems, Life Technologies, Paisley, UK), with ~10 ng of PCR products and 1.6 pmol of primer (forward or reverse) in each reaction. Sequencing products were cleaned by ethanol precipitation, and then separated by capillary electrophoresis on an ABI3130xl. Forward and reverse sequences were checked in Geneious v.6.1.2 (Kearse *et al*., 2012) to produce a consensus. Individual consensus sequences were aligned using CLUSTA LW v.2.0.12 (Thompson *et al*., 1994) through MEGA v.6 (Tamura *et al*., 2013). Sequences were then manually checked and trimmed, with alignments subsequently revised by eye to maximize positional homology.

Statistical analysis

In addition to the sequences obtained in this study, 52 COI sequences from sponge species were obtained from nucleotide database collection of GenBank with a search limited to Porifera, and a reference sequence from *S. lacustris*, optimizing for highly similar sequences (query = *S. lacustris* AND COI: date = 09.01.12). Sponge COI sequence data (from GenBank and obtained by sequencing) were aligned using CLUSTALW through MEGA5 and trimmed (553bp) to match the length of sequences obtained from GenBank. Pairwise sequence divergence was calculated among all pairs of species, genera, and families using Kimura's two parameter (K2P) model (Kimura, 1980), as in other barcoding studies (e.g. Shearer and Coffroth, 2008; Hebert *et al*., 2003a; Hebert *et al*., 2004a; Huang *et al*., 2008). All positions containing gaps and missing data were ignored. A distance table was processed to calculate divergence means (including standard error and ranges) within and between sponge families.

For UK sponge species, amino acid sequences were aligned using CLUSTALW. To quantify selection pressures on the COI locus the ratio of synonymous and non-synonymous (dS and dN) substitutions in COI sequences for

each species pair were estimated by the codon-based Z-test, with the variance computed using the analytical method (Nei and Gojobori, 1986), followed by a post-hoc sequential Bonferroni test (Rice, 1989).

Phylogenetic relationships among freshwater sponges were inferred from COI and ITS sequences using Neighbor-Joining (Saitou and Nei, 1987), with support for nodes calculated using a bootstrap test (Dopazo, 1994**;** Rzhetsky and Nei, 1992) with 500 replications. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al*., 2004) as the number of base substitutions per site. These units were also used to construct branch lengths. In some cases, groups of species that had diverged with bootstrap values <50% were condensed. *Corvomeyenia* sp. (Metaniidae) was used as an out-group as it appeared basal among Spongillida in former phylogenetic analyses (Addis and Peterson, 2005; Itskovich *et al*., 2007; Meixner *et al*., 2007; Redmond *et al*., 2007).

Results

Morphological analysis

Spicules from the UK survey (93 sponge samples) were examined. UK freshwater sponge species (*S. lacustris*, *E. mülleri*, *E. fluviatilis*, *R. ryderii* and *Eu. fragilis*) were recorded. Spicule morphological characteristics of each UK species are described (Table 1). Gemmules with gemmuloscleres were not present in every specimen. The presence of smooth oxea megascleres eliminated *E. mülleri* and *R. ryderii* but not *S. lacustris*, *E. fluviatilis*, or *Eu. fragilis* (see Chapter 2). Only samples of *S. lacustris* contained microscleres. Although, megascleres of *E. fluviatilis* were larger than megascleres of other species (Fig. 1), range values overlapped so there were no clear size classes to assist identification.

COI analysis

A total of 150 sponge colony sub-samples were sequenced. Sequencing of COI mtDNA was successful for sponges at 42 UK sites and eight others (from Germany, Ireland and Australia) (Supporting Information 4.1, Table S1). A total of 102 sequences were analysed representing 50 new sequences and 52 sequences from GenBank (Supporting Information 4.1, Table S2), and were aligned, then trimmed to

a 553 bp sequence. The COI sequence data represented 21 species belonging to 13 genera (in five families) and yielded the first COI data for *R. ryderii* (Fig. 2a) and *Eu. sinensis*. Sequences had a conserved primary structure. Aligned sequences from UK freshwater species produced in the present study displayed diagnostic SNP (single nucleotide polymorphism) in nine key places (Fig. 2a), enabling the differentiation of species after careful COI sequencing. Two of these nine (i.e. 22%) base pair differences are in replacement sites coding for different amino acid translations (Fig. 2a, b). The SNP from A to C at position 444, changes amino acid translation from methionine to leucine, and the SNP G to A at position 509 changes valine to isoleucinein in the translated protein.

Interspecific divergence

A total of 413 COI sequences (from sponge sub-samples and GenBank) were examined in this analysis. COI divergence ranged between 0.0% and 0.04% K2P and between 0.0% and 0.33% in marine sponges (Table 2). The average sequence divergence in:

(i) 378 interspecific freshwater sponge species comparisons is 0.01% (SE = 0.00%);
(ii) 14706 interspecific marine sponge species comparisons is 0.21% (SE = 0.00%).

The mean sequence divergence of 0.01% for freshwater species indicates that most pairs of COI sequences were separated by less than 1 diagnostic substitution in every 1000bp of their COI gene. COI sequences from UK populations were identical to those from other parts of Europe (Ireland and Germany). Negative values in substitution analysis of freshwater species indicate the number of replacement mutations is less than the number of silent mutations per silent site (dN<dS) (Castillo-Ramírez *et al*., 2011), with no significant positive values in non-synonymous substitutions (dN>dS) following post hoc tests (Supporting Information 4.1, Table S3).

Since some interspecific divergences were as low as 0%, threshold values typically implemented to differentiate between species (2–3%: Hebert *et al*., 2003b) could not be used. Additionally, implementing a threshold of 10 times the mean intraspecific variation for the group (Hebert *et al*., 2004b) (0.0007% mean intraspecific variation; 0.007% threshold), was also not appropriate since >80% of

sponge paired comparisons in this analysis exhibited genetic distances of 0% (Table 2). COI sequence divergence levels were low (<0.5%) for both freshwater and marine sponge species. COI sequence divergence values for marine species were higher than those in freshwater species, although intraspecific divergences were rarely > 0.1% (Table 2). Five marine sponge genera (*Suberites*, *Polymastia*, *Axinella*, *Scopalina*, and *Eurypon*) exhibited divergence levels >0.1%. As the intraspecific sequence divergence was at or near zero for other genera, separation of interspecific sequence divergence was not possible for sponge species (Fig. 3). COI sequence divergence levels between sponge genera (Fig. 4) displayed a similar pattern to levels between species, with low values of intra and interspecific variation. For the analyses of genera, freshwater sponge interspecific variation results were ≤0.1 (Fig 3 and 4). Comparisons of interspecific sequence divergence between sponge families illustrated a wider range of divergence values for marine sponges than freshwater sponges (Fig. 5a). However, intraspecific sequence divergences remained close to zero for both marine and freshwater sponge families (Fig. 5b).

Taxonomy of freshwater species

Although some bootstrap values were low (<50%), results from COI gene sequences supported the monophyly of freshwater sponges (Spongillina) with short distances between groups (Fig 6a). There was a low sum of branch lengths (0.081) inferring a low mutation rate in COI sequences. Although, internal relationships between the condensed species were unresolved, members of Lubomirskiidae formed a clade with some Spongillidae species (*Ephydatia* spp., *R. ryderii* and *Eunapius subterraneus*). There was a well-defined clade consisting of *S. lacustris* together with *Eunapius* species including *Eu. sinensis*. The exception was *Eunapius subterraneus* that instead clustered with *Ephydatia* sp, *Swartschewskia papyracea*, *B. dzegatajensis*, and *Ohridospongilla* sp. A cluster near the base of the tree consisted of members of Spongillidae (*Trochospongilla horrida, T. pennsylvanica* and *S. vastus*) with of members of Malawispongiidae (*Pachydictyum globosum* and *P. incrustans*). The amplified ITS sequences from five UK species and *Eu. sinensis* exhibited interspecific divergence, with the optimal tree (Fig. 6b) exhibiting similar phylogenetic relationships to the COI gene tree. Again, some internal relationships between the condensed species were unresolved (*Ephydatia* spp., *R. ryderii*).

However, in this analysis there was a well-defined separation between the sister groups of *S. lacustris* and *Eunapius* species.

Discussion

Aquatic biodiversity has been underestimated at all levels (Wörheide and Erpenbeck, 2007), with more species to be recognized and specimens to be identified than present levels of investigators can achieve using morphology (Will and Rubinoff, 2004). DNA barcoding (Hebert *et al*., 2003a, b) has been suggested as a way to overcome the issue of species identification. In particular, as specific characters for comparative morphology of sponges are scarce, prone to homoplasies, and are variable, unambiguous identification of sponge species using morphological methods is difficult (Wörheide and Erpenbeck, 2007). Using informative signature DNA sequences and a phylogenetic system might provide an opportunity to overcome these shortcomings (Wörheide and Erpenbeck, 2007). An important question addressed by this study concerned the variability of COI in freshwater sponges. Results and meta-analysis demonstrated the conservation of freshwater sponge COI sequences and low divergence in both freshwater and marine sponges. In the present study, both COI barcoding and spicule morphology have been used for species identification (Table 3) highlighting shortcomings in both approaches.

Other studies have demonstrated that COI is useful for the discrimination of closely related metazoan species over a broad range of taxonomic levels (e.g. Folmer *et al*., 1994; Bucklin *et al*., 2011). Mitochondrial DNA genes such as COI were proposed for barcoding in part, because they are easier to isolate, and there are many copy numbers in every cell (Radulovici *et al*., 2010). Similar to other protein coding genes, third codon position nucleotides in COI genes show a high incidence of base substitutions, leading to a rate of molecular evolution nearly three times greater than that of other genes (Bucklin *et al*., 2011). However, in the present study, low interspecific divergence (mean genetic distance =0.01%) prevented species being identified. Threshold values typically implemented to differentiate between species (2–3% genetic distance (K2P): Hebert *et al*., 2003b) could not be used with all freshwater sponge species, since some interspecific divergences were as low as 0%. Thus, it was not possible to set any threshold value that allows the exclusion of false

negatives (type II errors). Additionally, implementing a threshold of 10 times the mean intraspecific variation for the group (Hebert *et al*., 2004b) (0.0007% mean intraspecific variation; 0.007% threshold), was also not appropriate since >80% of sponge paired comparisons in this analysis exhibited genetic distances of 0%. Analysis of COI from both marine and freshwater species suggests an upper limit of 0.34±0.07 % genetic distance (K2P) for mean intraspecific divergence in sponge taxa. This is also lower than suggested threshold values. Low COI sequence divergences found in the present study support other research (Duran *et al*., 2004; Wörheide, 2006; Solé-Cava and Worheide, 2007; Meixner *et al*., 2007) including one base transition resulting in a synonymous substitution in COI gene of Mediterranean and Atlantic *Crambe crambe* populations. The present work updates these data and includes additional freshwater sequences. Low COI sequence divergences have been quantified in phylum Porifera. For comparison, in other invertebrate studies, moths have demonstrated an average COI sequence divergence of 6.5%, and an average of 16.4% in spiders (Barrett and Hebert, 2005). Although thresholds can be effective in screening for substantially divergent novel taxa (Meyer and Paulay, 2005), the use of thresholds for sponge species delineation is limited, due to the absence of a clear barcoding gap.

Together, these data suggest the COI locus is conserved in sponges. Instead of a COI barcoding gap, diagnostic SNP (single nucleotide polymorphism) at 9 key sites enabled UK species to be identified. UK freshwater sponge sequences exhibited mostly synonymous site variation (dN<dS), with silent mutations in amino acids rather than replacement mutations consistent with neutral evolution (López-Pérez *et al*., 2014). Previous DNA barcoding studies in Lake Tanganyika sponges and in Anthozoa have found similar patterns (Erpenbeck *et al*., 2011; Lavrov *et al*., 2005; Hellberg, 2006) and suggested that synonymous substitution rates in these basal metazoan groups are about 100 times slower than for Bilateria. Slow-evolving mtDNA may be linked to the presence of an excision repair system absent in other animal mitochondria (Hebert *et al*., 2003). A slow rate of mtDNA evolution is also found in most plant groups (Zhang and Hewitt, 2003). This variability in the rate of evolution among taxa indicates that no standard level of sequence divergence can be implemented to discern species (Will and Rubinoff, 2004).

A more variable 440 bp downstream fragment was added to the standard COI barcoding gene as part of the Sponge Barcoding Project assessment using specific primers (Erpenbeck, *et al*., 2006) (Supporting Information 4.1, Fig. S3a). However, this fragment was not added to sequence data in the present investigation as specific primers suggested were not successful in amplifying UK freshwater samples. Instead the present investigation concentrated on using the standard COI barcoding fragment with "universal" primers (Fromer *et al*., 1994). This allowed comparison with other freshwater sponge sequences (see Addis and Peterson, 2005) and calculations of K2P distances. Although the use of K2P as a divergence measurement has been suggested to be inappropriate for barcoding analysis (Srivathsana and Meira, 2012) with p-distances suggested as an alternative (Nei and Kumar, 2005), K2P comparison in the present work is consistent with other barcoding studies with other invertebrates e.g. (Shearer and Coffroth, 2008; Hebert *et al*., 2003a; Hebert *et al*., 2004a; Huang *et al*., 2008; Barrett and Hebert, 2005; Hajibabaei *et al*., 2006) and is appropriate for COI sequence data with low divergence distances (Nei and Kumar, 2005).

In addition to COI sequences, alternative loci considered for sponge molecular systematics have been the 18S and the 28S rDNA genes. In other research, Lopp *et al*. (2007) demonstrated that the D3 region of the 28S rDNA gene displays species specific variation and may be a suitable marker for freshwater sponge identification (see Chapter 2). Species sequence pairs with COI intraspecific divergence values > 0.1% include members from Suberitidae and Polymastiidae that, using the 28S rDNA are suggested to be in adjacent clades (Morrow *et al*., 2012). However, in order to make hypothesis on phylogenetic relationships within these families more sequence data are needed in an integrated approach with morphological character traits. Attempts to amplify another rRNA gene, 18S rDNA, (as outlined in: Peterson and Addis, 2000) in preliminary work were not successful and resulted in double bands on PCR gels. In any case, discrimination between sponge species based on 18S rDNA has been shown to be less informative than COI due to small number of variable sites (Addis and Peterson 2005). More informative results are obtained when several genes are used and congruent patterns appear (e.g.

Addis and Peterson, 2005; Redmond *et al*., 2007; Itskovich *et al*., 2007). The use of internal transcribed spacers (ITS) of rDNA gene sequences separating conserved regions of the rDNA genes was suggested in SBP protocol to optimise species differentiation, and this was confirmed with UK species (and *Eu. sinensis*). Although problems with intra-genomic polymorphisms in ITS rDNA sequences have been recorded (Wörheide *et al*., 2004), these sequences have been used in other sponge research to resolve phylogenetic relationships (Erpenbeck *et al*., 2011; Itskovich *et al*., 2008; Meixner *et al*., 2007). The resolution of the ITS tree (Fig. 6b) seems to be higher than that obtained with COI data, illustrating separation of members of *Eunapius* clade from *S. lacustris*.

Before COI barcoding techniques, traditional methods of taxonomy used morphology as key factors in describing and naming species (Packer *et al*., 2009). However, variation in body sponge shape (see Chapter 2), and environmentally induced spicule variation (see Chapter 5) can lead to incorrect species identification. Instead, the presence of gemmules and their morphological traits are diagnostic characters in freshwater sponge taxonomy (Manconi and Pronzato, 2002). However gemmules are not present in all species (e.g. *Pachydictyum spp*). Also, gemmules are not present at all life history stages so transformation of adult sponge tissue to gemmules may be required. In the absence of gemmules, megascleres in three UK freshwater species may be confused by the presence of smooth oxeas (*S. lacustris, E. fluviatilis* and *Eu. fragilis*, Table 1) of an overlapping size range (Fig. 1). Megasclere similarity may reflect recent common ancestry and/or also adaptive similarities due to convergent evolution of smooth megascleres (Hansen, 1997). These species may co-exist (see Chapter 2) raising the possibility that slides produced for taxonomic purposes may have megascleres from more than one species. For example, although microscope slides with *S. lacustris* spicules may have microscleres, any smooth megascleres could also be from *Eu. fragilis* as these species can co-exist (see Chapter 2). In the present study, this issue was overcome by collecting colony sub-samples, but in mixed samples it will reduce the usefulness of traditional taxonomic keys. In this example of megasclere similarity, barcoding outperforms morphological taxonomic methods by incorporating a complex set of data to identify sponge diversity (Packer *et al*., 2009). Using genetic sequencing

methods provides taxonomic references to confirm identiy in instances of morphological similarity/plasticity and with sampling inconsistency.

By mapping gemmule morphology on COI and ITS molecular trees (Fig. 6a, b), results suggest that using only gemmular traits reflects relationships among only some freshwater sponge taxa (Harcet *et al.*, 2010). For example, gemmules of *Eu. subterraneus* are tubeless, while gemmule foramina in the genus *Eunapiu*s are tubular (Penney and Racek, 1968). This morphological difference, together with COI data, suggest *Eu. subterraneus* is distinct from other members of *Eunapius* clade (Harcet *et al.*, 2010). Reduction or loss of gemmules is also found in families Lubomirskiidae, Malawispongiidae, and Metschnikowiidae. Although bootstrap values were low, Lubomirskiid sponges from the Lake Baikal region, clustered with a cosmopolitan species *E. fluviatilis* (Fig. 6a), supporting the suggestion that species belonging to Lubomirskiidae may have evolved from *Ephydatia* (Addis and Peterson, 2005; Meixner *et al*., 2007; Erpenbeck *et al*., 2011). For the first time both COI and ITS sequence data (Fig. 6a, b) have revealed the presence of *R. ryderii* in this cluster. Additionally, despite spicule variability, *R. ryderii* has birotulate gemmuloscleres (see Chapter 6) with a morphological structure similar to those found in *E. fluviatilis* and *E. mülleri*, suggesting this amphiatlantic species may also have evolved from *Ephydatia*. Species of Metaniidae, at the base of the Spongillida (Itskovich *et al*., 2007; Meixner *et al*., 2007) are widely distributed and can produce gemmules. This raises the possibility that a common ancestor of freshwater sponges may have been able to produce gemmules, and that this trait may have been lost in other species (e.g. *Pachydictyum spp*.) especially those that are located in stable, permanent habitats (Itskovich *et al*., 2007). Some marine sponge taxa (e.g. *Haliclona*) also have gemmules and a similar life cycle to Spongillidae (Pronzato and Manconi, 1994) supporting this possibility.

The present study suggests that the use of morphological data as part of a combined analysis with molecular data may assist in species identification, together with a better understanding of phylogenetic relationships. DNA barcoding represents a useful tool for taxonomy in situations where gemmules are absent, but without the integration of traditional morphological approaches species discrimination is not

always achievable (Table 2). Basal metazoans need both careful sequence data on diagnostic SNPs rather than % divergence, combined with morphological data (see Lopp *et al*., 2007; Ferri *et al*., 2009). Results in the present study enable freshwater sponge samples collected in different localities, in different studies, or from Museum collections, to be identified in a way that arbitrary designations (e.g. just labelling samples 'sponges') do not. One consequence of barcoding is that it may increase the motivation to collate and organize biodiversity data (Janzen *et al*., 2005). This is important as there is an incomplete picture of overall sponge diversity (Manconi and Pronzato, 2008). Continued efforts to increase the number of taxa and genes sampled from Spongillidae found in a wide range of habitats may reveal a new picture of diversity incorporating both genetic and morphological data.

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Table 1. Spicule (megasclere, microsclere and gemmulosclere) characteristics facilitate identification of five UK freshwater sponge species (Spongillidae) (see Chapter 2).

	Megasclere		Micro-sclere	Gemmulo-sclere
	Image (▬▬▬ = 100µm)	Description		
S. lacustris		Slightly curved, smooth oxea.	Slightly curved oxea with micro-spines	Slightly to strongly curved spined oxea.
Eu. fragilis		Slightly curved, smooth oxea.	None	Slightly curved or straight spined oxea.
E. fluviatilis		Slightly curved, smooth oxea.	None	Birotules with occasionally spined shafts and >10 rays.
E. mülleri		Slightly curved oxea, with micro-spines.	None	Birotules with a shorter shaft than *E. fluviatilis* and <10 rays.
R. ryderii		Variable spined or micro-spined oxea.	None	Birotules and/or pseudo-birotules where length > maximum width.

Table 2. Mean intraspecific evolutionary divergence over sequence pairs within marine and freshwater sponge families. The number of base substitutions per site from averaging over all sequence pairs within each genus is shown using the Kimura 2 - parameter model. Standard error shown when number of sequences >3.

	Sponge family	Genus (sample size)	Mean divergence ± S.E.		
			> 0.10	0.01 – 0.10	0.00 – 0.01
Freshwater	Spongillidae	*Eunapius* (21)			0.01 ± 0.00
		Spongilla (24)			0.00 ± 0.00
		Ephydatia (28)			0.00 ± 0.00
		Racekiela (8)			0.00 ±0.00
		Trochospongilla(2)			0.01
	Lubomirskiidae	*Baikalospongia* (7)			0.00 ± 0.00
		Lubomirskia (4)			0.00 ± 0.00
		Swartschewskia(2)			0.01
	Malawispongi-dae	*Pachydictyum* (2)			0.00
	Potamolepidae	*Oncosclera* (2)			0.00
Marine	Suberitidae	*Suberites* (12)	0.34 ± 0.07		
	Polymastiidae	*Polymastia* (3)	0.20 ± 0.01		
	Axinellidae	*Axinella*(2)	0.19		
	Dictyonellidae	*Scopalina* (13)	0.15 ± 0.01		
		Svenzea (2)			0.00
	Raspailiidae	*Eurypon* (2)	0.25		
		Raspailia (2)			0.01
	Tethyidae	*Tethya* (22)		0.10 ± 0.00	
	Chalinidae	*Haliclona* (17)		0.06 ± 0.02	
	Ancorinidae	*Stelletta* (4)		0.06 ± 0.02	
		Rhabdastrella (2)		0.07	
	Tedaniidae	*Tedania* (17)		0.02 ± 0.00	
	Tetillidae	*Craniella* (4)		0.05 ± 0.02	
		Cinachyrella (10)		0.05 ± 0.00	
		Tetilla (2)		0.07	
	Callyspongiidae	*Callyspongia* (14)		0.04 ± 0.08	
	Phloeodictyidae	*Oceanapia* (2)		0.06	
	Geodiidae	*Geodia* (7)		0.08 ± 0.01	
		Erylus (3)		0.07 ± 0.02	
		Pachymatisma (18)			0.00 ± 0.00
	Clionidae	*Cliona* (36)		0.05 ± 0.00	
		Pione (3)		0.03 ± 0.01	
		Clionaopsis (2)			0.00
	Desmacellidae	*Biemna* (3)		0.10 ± 0.01	
		Neofibularia (2)			0.00
	Halichondriidae	*Halichondria* (10)		0.05 ± 0.01	
		Hymeniacidon (8)			0.00 ± 0.00

	Petrosiidae	*Petrosia* (9)		0.09 ± 0.01	
		Xestospongia (17)			0.00 ± 0.00
	Ianthellidae	*Hexadella* (6)		0.03 ± 0.00	
		Ianthella (6)			0.00 ± 0.00
	Geodiidae	*Pachymatisma* (18)			0.00 ± 0.00
	Aplysinidae	*Aplysina* (18)			0.00 ± 0.00
	Coelosphaeri-dae	*Lissodendoryx* (4)			0.00 ± 0.00
	Microcionidae	*Clathria* (3)			0.00 ± 0.00
		Microciona (3)			0.00 ± 0.00
		Artemisina (2)			0.00
		Holopsamma (2)			0.00
	Chrondrillidae	*Chondrilla* (2)			0.00
	Heteroxyidae	*Halicnemia* (2)			0.00
	Iotrochotidae	*Iotrochota* (2)			0.00
	Podospongiidae	*Negombata* (2)			0.00
	Placospongiidae	*Placospongia* (2)			0.01
	Hemiasterelli-dae	*Stelligera* (2)			0.00

Table 3. Suggested steps in the identification of freshwater sponges. Genetic data can serve as a tool to determine species identity, in both morphological and molecular investigations. DNA sequences are obtained in both types of investigation in, but phylogenetic studies may require additional sequence data.

	Morphological identification	**Sequence data**	**Molecular identification**
1	Collect specimens and/or museum samples	3a. Obtain good quality DNA	Collect specimens and/or museum samples
2	Examine spicule morphology in detail	3b. Sequence portion of DNA	Microscopic removal of visible epibionts
3	Determine species →	3c. Run software to obtain trees or similarity values	← Determine species
4	Check available earlier names if necessary	3d. Check for congruence of DNA and morphology	Sequence several key genes. Edit sequences to obtain a high quality read.
5	Describe each specimen	3e. Draw preliminary taxonomic conclusions	Run software to obtain trees. Interpret trees.

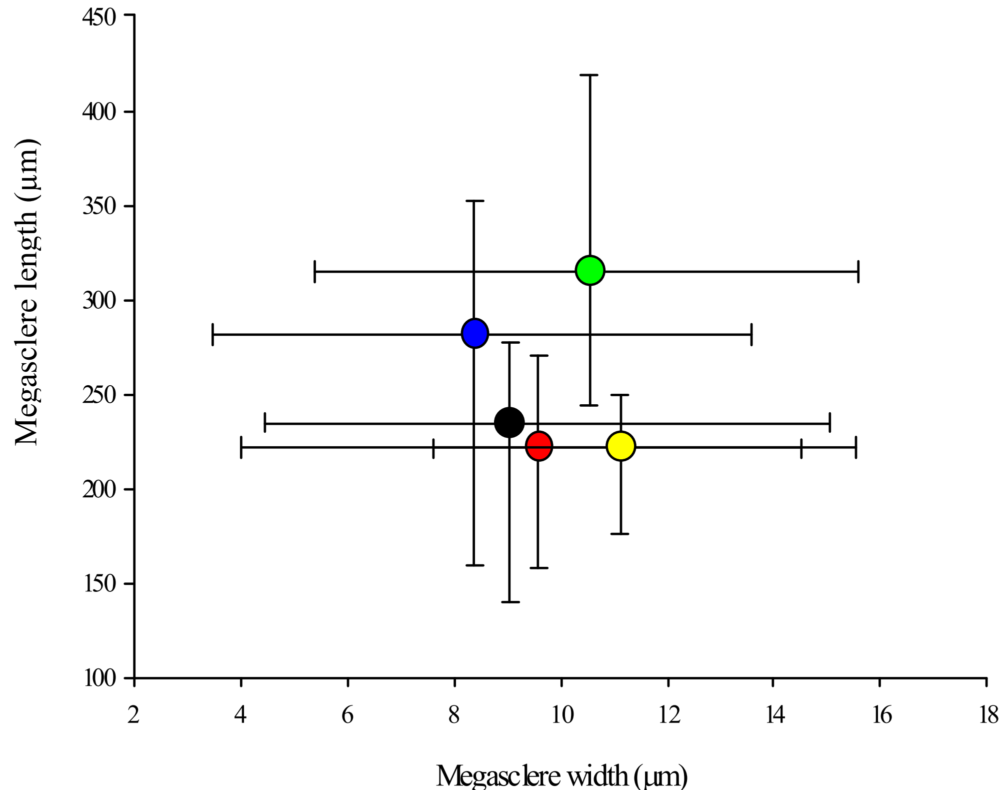


Fig. 1. Average megasclere length and width of five UK freshwater sponge species with range of values. Green = *E. fluviatilis*; blue = *R. ryderii*; black = *S. lacustris*; red = *E. mülleri*; yellow= *Eu. fragilis*.

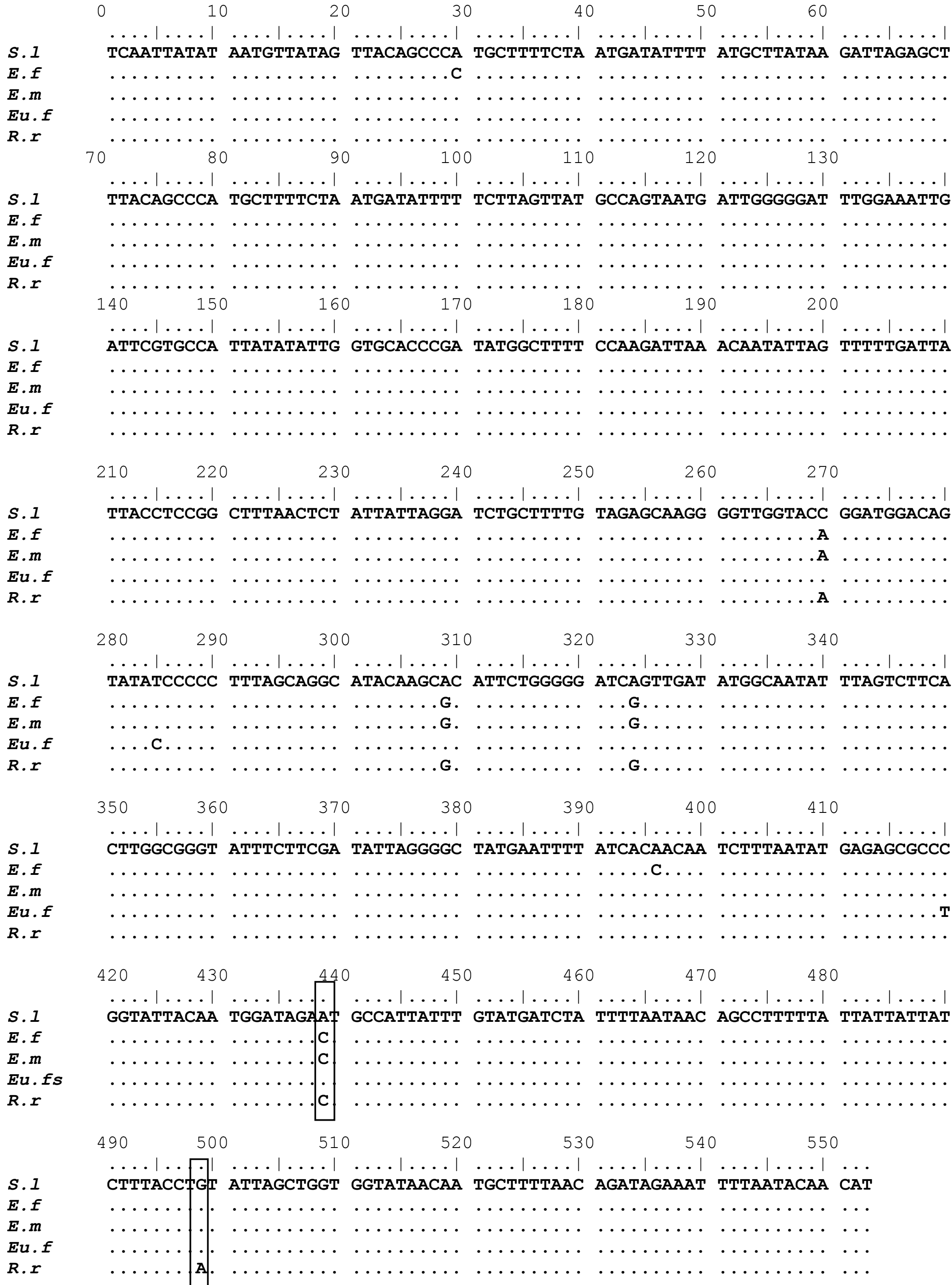


Fig.2.a. Alignment of a 553 bp fragment of the 5′ end of the COI gene from *S. lacustris*, *E. fluviatilis*, *E. mülleri*, *Eu. fragilis*, and *R. ryderii*. Identities have been indicated by dots. Boxes indicate sites where polymorphisms result in different amino acid translations.

S. lacustris	M	L	I	R	L	E	L	S	A	P	G	S	M	L	G	D	D	Q	L	Y
E. fluviatilis	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
E. mülleri	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Eu.fragilis	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
R. ryderii	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

S. lacustris	N	V	I	V	T	A	H	A	F	L	M	I	F	F	L	V	M	P	V	M
E. fluviatilis	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
E. mülleri	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Eu.fragilis	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
R. ryderii	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

S. lacustris	I	G	G	F	G	N	W	F	V	P	L	Y	I	G	A	P	D	M	A	F
E. fluviatilis	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
E. mülleri	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Eu.fragilis	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
R. ryderii	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

S. lacustris	P	R	L	N	N	I	S	F	W	L	L	P	P	A	L	T	L	L	L	G
E. fluviatilis	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
E. mülleri	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Eu.fragilis	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
R. ryderii	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

S. lacustris	S	A	F	V	E	Q	G	V	G	T	G	W	T	V	Y	P	P	L	A	G
E. fluviatilis	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
E. mülleri	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Eu.fragilis	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
R. ryderii	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

S. lacustris	I	Q	A	H	S	G	G	S	V	D	M	A	I	F	S	L	H	L	A	G
E. fluviatilis	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
E. mülleri	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Eu.fragilis	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
R. ryderii	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

S. lacustris	I	S	S	I	L	G	A	M	N	F	I	T	T	I	F	N	M	R	A	P
E. fluviatilis	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
E. mülleri	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Eu.fragilis	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
R. ryderii	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

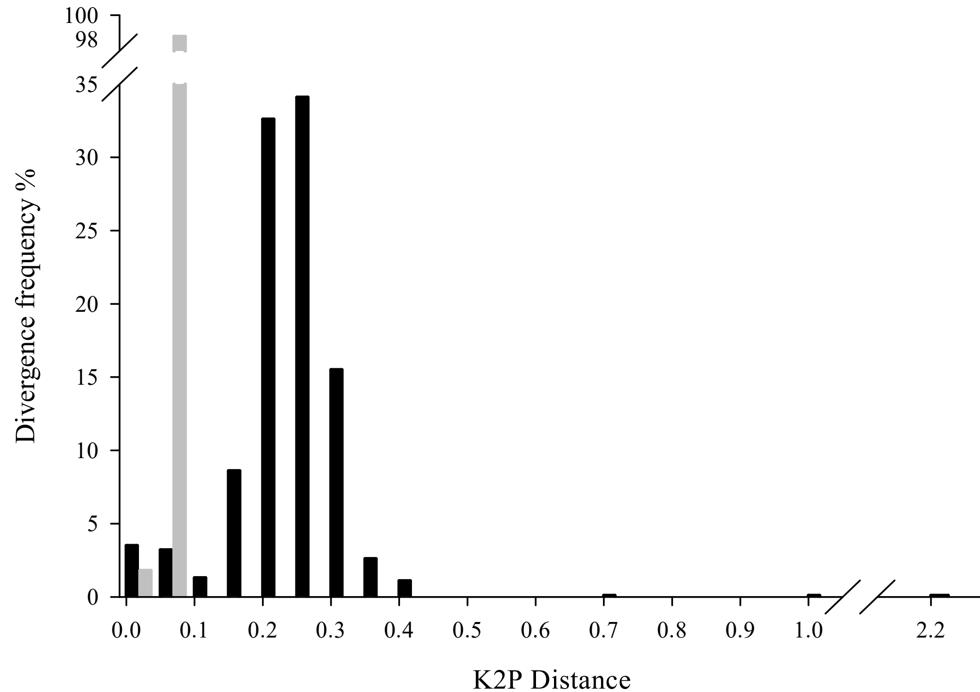
S. lacustris	G	I	T	M	D	R	M	P	L	F	V	W	S	I	L	I	T	A	F	L
E. fluviatilis	.	.	.	.	.	.	L	.	.	.	.	.	.	.	.	.	.	.	.	.
E. mülleri	.	.	.	.	.	.	L	.	.	.	.	.	.	.	.	.	.	.	.	.
Eu.fragilis	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
R. ryderii	.	.	.	.	.	.	L	.	.	.	.	.	.	.	.	.	.	.	.	.

S. lacustris	L	L	L	S	L	P	V	L	A	G	G	I	T	M	L	L	T
E. fluviatilis	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
E. mülleri	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Eu.fragilis	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
R. ryderii	.	.	.	.	.	.	I	.	.	.	.	.	.	.	.	.	.

S. lacustris	D	R	N	F	N	T	T
E. fluviatilis	.	.	.	.	.	.	.
E. mülleri	.	.	.	.	.	.	.
Eu.fragilis	.	.	.	.	.	.	.
R. ryderii	.	.	.	.	.	.	.

Fig.2.b. Alignment of amino acid sequence from COI gene from *E. fluviatilis*, *E. mülleri*, *Eu. fragilis*, *S. lacustris* and *R. ryderii*. Identities have been indicated by dots. Where G=glycine, P=proline, A =alanine, V=valine, L=leucine, I=Isoleucine, M= methionine, F=phenylalanine, Y= tyrosine, W= tryptophan, H= histidine, R= arginine, Q= glutamine, N= asparagines, E= glutamic acid, D= aspartic acid, S=serine, T= threonine.

a)



b)

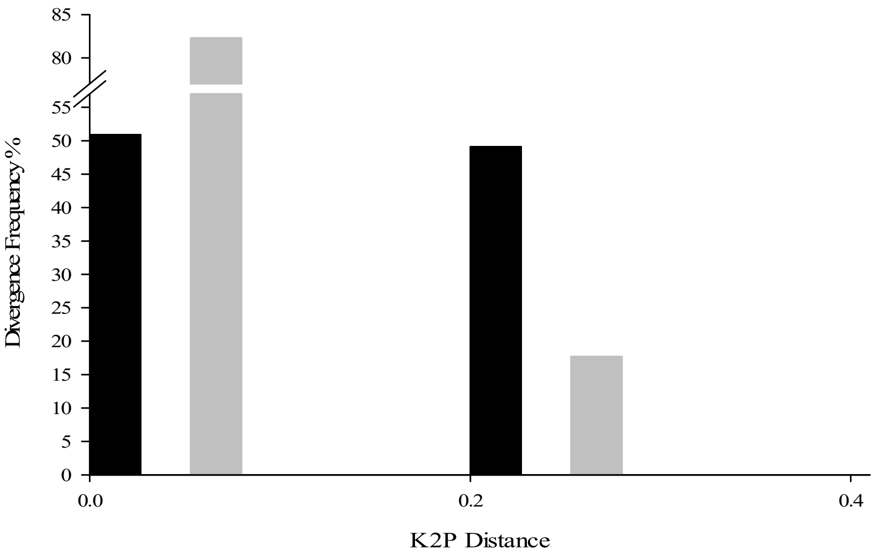
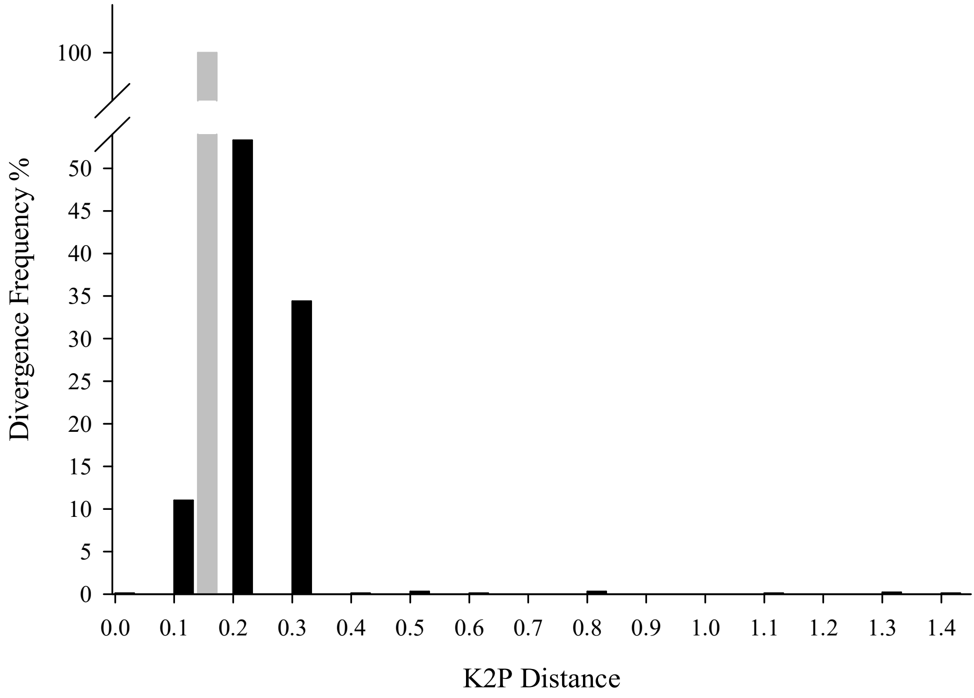


Fig.3. Comparison of interspecific COI divergence a) interspecific and b) intraspecific freshwater (shown in grey) and marine (shown in black) sponge species. Divergences were calculated using Kimura's two parameter (K2P) model (Kimura, 1980).

a)



b)

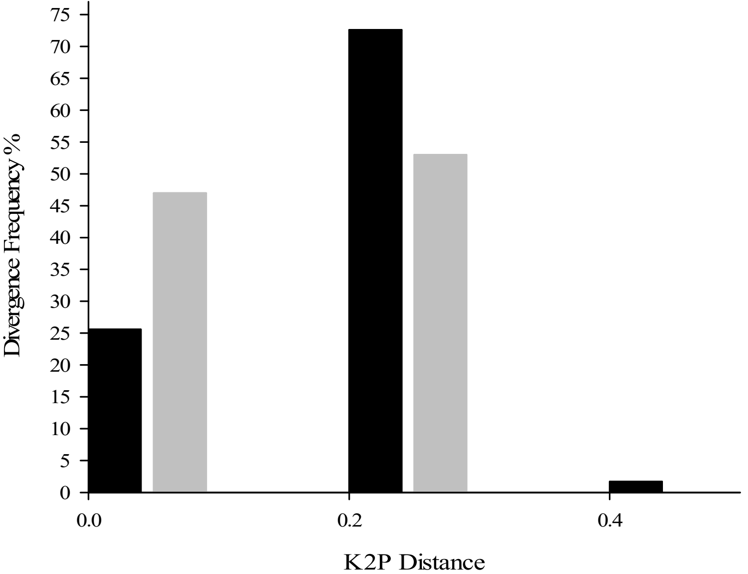
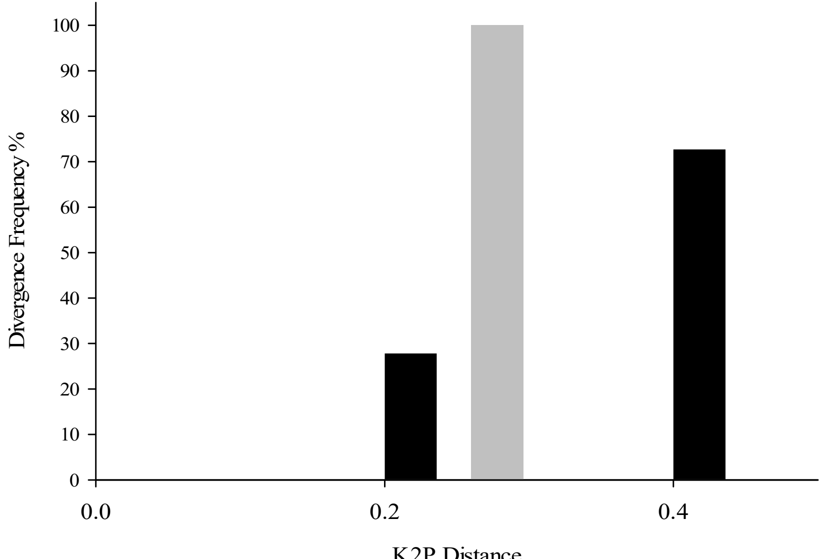


Fig.4. Comparison of interspecific COI divergence a) interspecific and b) intraspecific freshwater (shown in grey) and marine (shown in black) sponge genera. Divergences were calculated using Kimura's two parameter (K2P) model (Kimura, 1980).

a)



b)

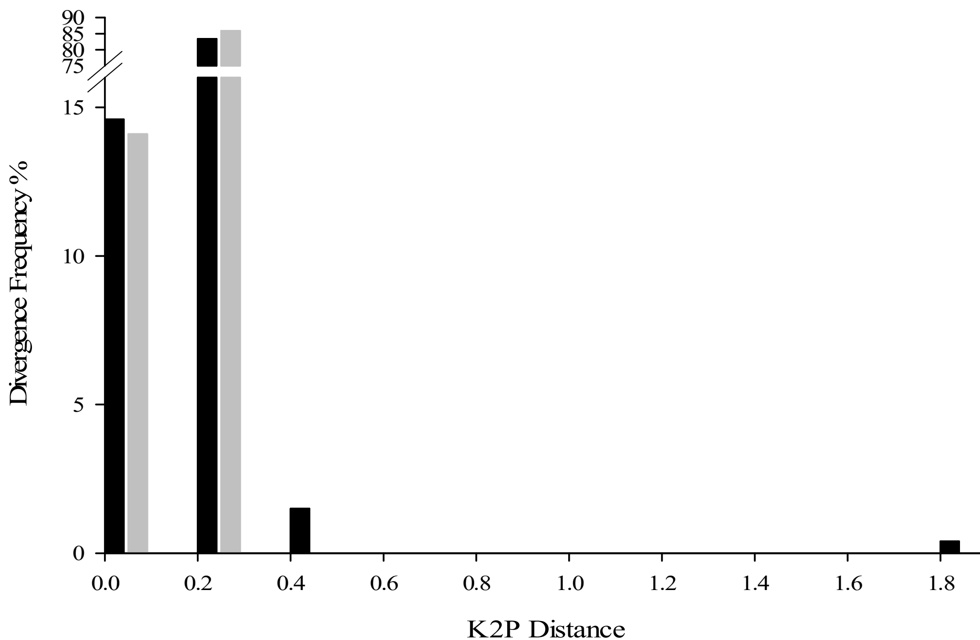


Fig. 5. Comparison of interspecific COI divergence a) a) interspecific and b) intraspecific freshwater (shown in grey) and marine (shown in black) sponge families. Divergences were calculated using Kimura's two parameter (K2P) model (Kimura, 1980).

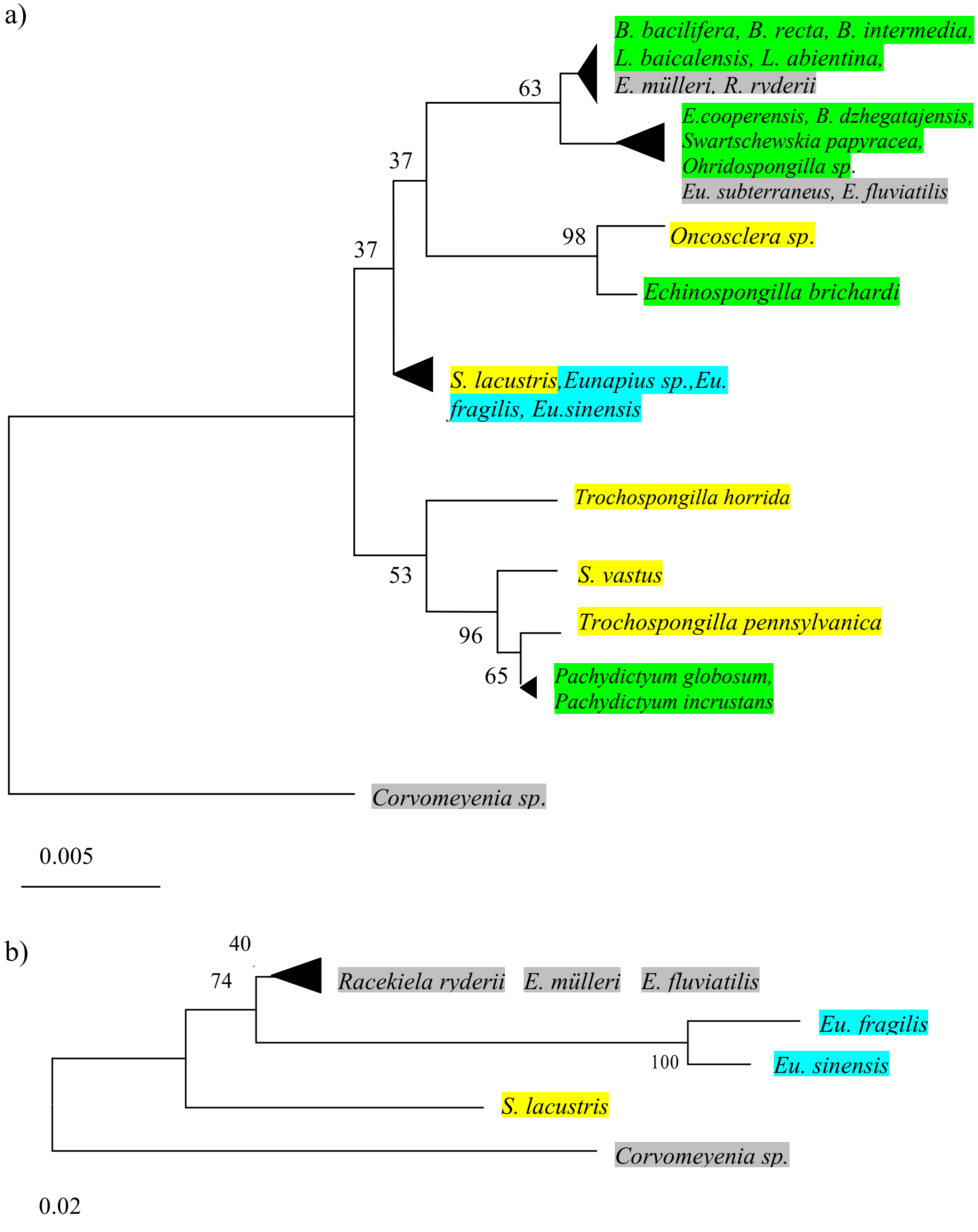


Fig. 6. Phylogenetic relationships among freshwater sponge taxa based on alignment of a) COI and b) ITS sequences both with gemmule characteristics highlighted. The bootstrap values are indicated in the tree. Genus names *Baikalospongi*a, *Lubomirskia*, *Ephydatia*, *Eunapius*, *Racekiela* and *Spongilla* have been abbreviated. Key to gemmule characteristics: green = reduction/loss of gemmules; grey= gemmules with a simple foramen (see Chapter 1); yellow = gemmules with a slightly elevated/conical foramen; blue = gemmules with a tubular foramen.

Supporting Information 4.1.

Table S1. Sites (n=50) where sponge were present with habitat type, latitude and longitude.

	Lat	Long	Habitat	Site Code	Site Name
1	53.105	-2.574	Canal	SUN100	Bremilow Bridge
2	53.172	-2.816	Canal	SUN119	Egg.Bridge Waverton
3	53.282	-2.889	Canal	SUN145	Stanlow Bridge
4	53.069	-2.575	Canal	LLE015	Wrenbury Heath Bridge
5	52.914	-2.805	Canal	LLE048	Bettisfield Bridge
6	52.904	-2.818	Canal	LLE050	Hampton Bank Bridge
7	52.9	-2.876	Canal	LLE057	Ellesmere Tunnel
8	52.89	-2.99	Canal	LLW011	Hindford
9	52.927	-3.055	Canal	LLW019	Gledrid Bridge
10	52.972	-3.127	Canal	LLW040	Plas Ifan
11	52.984	-3.182	Canal	LLW048	Pentrefelin Bridge
12	52.818	-3.030	Canal	MGN080	Spiggots Bridge
13	52.817	-3.033	Canal	MGN082	Gronwyn Bridge
14	52.78	-3.095	Canal	MGS093	Walls Bridge
15	52.76	-3.09	Canal	MGS100	Four Crosses
16	52.601	-3.194	Canal	MGS128	Long Bridge
17	52.562	-3.228	Canal	MGS143	Glanhafren
18	53.362	-2.602	Canal	BDW012	Grappenhall, Walton
19	53.552	-2.169	Canal	RCD072	Oldham Road Bridge
20	53.527	-2.16	Canal	RCD076	Chadderton
21	53.509	-2.158	Canal	RCD078	Failsworth
22	53.27	-2.539	Canal	TME200	Soote Bridge, Anderton
23	53.554	-2.375	Canal	MBN014	Little Lever, Prestolee
24	53.556	-2.378	Canal	MBN015	Little Lever, Crompton
25	50.689	-3.488	Canal	EXT001	Countess Wear Bridge, Exeter
26	51.762	-1.270	Canal	NHM133	Oxford Canal Rugby
27	51.642	-3.027	Canal	MNB040	Ty Coch Cwmbran
28	51.655	-0.425	Canal	GUC001	GUC Rickmansworth
29	52.686	-1.102	Canal	NHM070	GUC Leicester Line
30	51.647	-3.825	Canal	NTT001	Neath junction rail bridge
31	54.100	-2.165	Lake	MHT001	Malham Tarn
32	53.015	-4.016	Lake	LLL001	Llyn Llagi
33	54.430	-3.262	Lake	BMT001	Burnmoor Tarn
34	53.072	-4.151	Lake	LLC001	Llyn Cwellyn
35	54.41	-2.890	Lake	NHM020	Windermere, Lake District
36	51.418	-0.999	Lake	NHM110	Searles Farm, Reading
37	51.590	-0.046	Lake	NHM121	Walthamstow Waterworks
38	51.590	-0.046	Lake	NHM123	Walthamstow Waterworks
39	54.372	-2.991	Lake	NHM140	Priest's Pot, Lake District
40	54.543	-7.989	Lake	MAL001	Mallybreen Lough, Northern Ireland
41	51.38	-0.954	River	NHM030	River Blackwater, Reading
42	51.380	-0.954	River	NHM040	River Blackwater, Reading
43	50.914	-3.290	River	CLM001	River Culm
44	51.873	-9.584	River	ROI010	River Sheen, Ireland
45	52.236	-7.210	River	ROI075	River Mahon, Ireland
46	52.644	-7.230	River	ROI093	River Nore, Ireland
47	52.920	-7.359	River	ROI100	River Erkina, Ireland
48	50.315	7.628	River	GER001	Koblens, Germany
49	-25.359	151.917	River	PDA001	Paradise Dam, Australia
50	51.462	-3.166	Brackish	CBW001	Cardiff Bay

Table S2. Freshwater sponge species included in the study and corresponding GenBank accession numbers of all analysed sequences.

Family	Species	GenBank accession no.
Lubomirskiidae	*Baikalospongia bacillifera*	DQ176780.1, DQ167167.1, EU000570.1
	Baikalospongia dzhegatajensis	EF025856.1
	Baikalospongia intermedia	EU000567.1, DQ167168.1
	Baikalospongia recta	EU000569.1
	Lubomirskia abietina	DQ167170.1
	Lubomirskia baicalensis	EU000568.1, GU385217.1, DQ167169.1
	Swartschewskia papyracea	EU000571.1 , DQ167171.1
	Rezinkovia echinata	NC018360.1
Spongillidae	*Ephydatia cooperensis*	DQ087505.1
	Ephydatia fluviatilis	DQ176777.1, JF340437.1, GQ411060.1, DQ167174.1
	Ephydatia mülleri	DQ176778.1, GQ411061.1, EU237481.1, DQ167172.1, AJ843884.1, GQ411062.1
	Ephydatia sp.	DQ167173.1
	Eunapius fragilis	DQ176779.1, AJ843882.1
	Eunapius sp.	JF816200.1, JF816204.1, JF816210.1 , JF816205.1 JF816211.1, JF816208.1 DQ167175.1
	Eunapius subterraneus	GU086203.1, FJ715439.1
	Spongilla lacustris	AJ843883.1, HQ379431.1, DQ167176.1 EU000572.1
	Spongilla vastus	DQ167180.1, DQ167166.1
	Trochospongilla horrida	EF025854.1
	Trochospongilla pennsylvanica	DQ087503.1
Malawispongiidae	*Pachydictyum globosum*	DQ167177.1
	Pachydictyum incrustans	DQ167178.1
Metaniidae	*Corvomeyenia sp.*	DQ176781.1
Potamolepidae	*Oncloslera sp.*	JF816201.1
	Echinospongilla brichardi	EU000573.1
Incertae sedis	*Ohridospongilla sp.*	EF025855.1
	Spongillina sp.	JF816207.1

Table S3. Codon-based test of positive selection for analysis between COI sequences from freshwater sponge species indicating the probability of rejecting the null hypothesis (dN = dS) in favour of the alternative hypothesis (dN > dS) (below diagonal), with significant value (*p*<0.05) in bold. Post hoc sequential Bonferroni tests (Rice, 1989) indicated that these were not significant. The test statistic (dN - dS) is above the diagonal.

B.bacillifera		-1.8	-1.0	-1.0	-1.9	-1.4	-1.8	-1.8	-1.0	-1.8	-1.9	-2.1	-2.4	-1.0	-1.0	-2.1	-2.0	-2.6	-2.8	-2.4	-2.4	-1.9	-1.8	-2.2	-2.8	-2.2	-1.0	-0.7
B.dzhegatajensis	1		-1.4	-1.4	-2.2	-1.0	0.0	0.0	-1.4	-1.4	-2.1	-2.4	-2.6	-2.0	-1.4	-2.1	-1.0	-2.6	-2.8	-2.4	-2.4	-2.1	0.0	-2.0	-2.8	-2.2	-1.4	-1.2
B.intermedia	1	1		0.0	-1.7	-1.0	-1.4	-1.4	0.0	-1.4	-1.5	-1.9	-2.1	-1.4	0.0	-1.8	-1.8	-2.4	-2.6	-2.1	-2.2	-1.5	-1.4	-2.0	-2.6	-2.0	0.0	1.0
B. recta	1	1	1		-1.7	-1.0	-1.4	-1.4	0.0	-1.4	-1.5	-1.9	-2.1	-1.4	0.0	-1.8	-1.8	-2.4	-2.6	-2.1	-2.2	-1.5	-1.4	-2.0	-2.6	-2.0	0.0	1.0
Corvomeyenia_sp.	1	1	1	1		-1.9	-2.2	-2.2	-1.7	-2.2	-1.6	-1.8	-2.1	-2.2	-1.7	-2.1	-2.4	-2.3	-2.5	-2.3	-2.2	-1.6	-2.2	-2.2	-2.1	-2.2	-1.7	-1.6
Ephydatia sp.	1	1	1	1	1		-1.0	-1.0	-1.0	-1.0	-1.9	-2.1	-2.4	-1.8	-1.0	-2.1	-1.4	-2.6	-2.8	-2.4	-2.4	-1.9	-1.0	-2.0	-2.8	-2.2	-1.0	-0.7
E.cooperensis	1	1	1	1	1	1		0.0	-1.4	-1.4	-2.1	-2.4	-2.6	-2.0	-1.4	-2.1	-1.0	-2.6	-2.8	-2.4	-2.4	-2.1	0.0	-2.0	-2.8	-2.2	-1.4	-1.2
E.fluviatilis	1	1	1	1	1	1	1		-1.4	-1.4	-2.1	-2.4	-2.6	-2.0	-1.4	-2.1	-1.0	-2.6	-2.8	-2.4	-2.4	-2.1	0.0	-2.0	-2.8	-2.2	-1.4	-1.2
E.mülleri	1	1	1	1	1	1	1	1		-1.4	-1.5	-1.9	-2.1	-1.4	0.0	-1.8	-1.8	-2.4	-2.6	-2.1	-2.2	-1.5	-1.4	-2.0	-2.6	-2.0	0.0	1.0
Eu.subterraneus	1	1	1	1	1	1	1	1	1		-2.1	-2.4	-2.6	-2.0	-1.4	-2.3	-1.8	-2.8	-3.0	-2.6	-2.7	-2.1	-1.4	-2.2	-3.0	-2.4	-1.4	-1.2
Eu.sinensis	1	1	1	1	1	1	1	1	1	1		-1.0	-1.4	-2.1	-1.5	-2.0	-2.4	-2.3	-2.5	-2.3	-2.1	0.0	-2.1	-1.9	-2.5	-2.1	-1.5	-1.3
Eunapius sp	1	1	1	1	1	1	1	1	1	1	1		-1.8	-2.4	-1.9	-2.2	-2.6	-2.5	-2.7	-2.5	-2.4	-1.0	-2.4	-2.1	-2.7	-2.4	-1.9	-1.7
Eu.fragilis	1	1	1	1	1	1	1	1	1	1	1	1		-2.6	-2.1	-2.4	-2.8	-2.7	-2.9	-2.7	-2.6	-1.4	-2.6	-2.4	-2.9	-2.6	-2.1	-2.0
L.abietina	1	1	1	1	1	1	1	1	1	1	1	1	1		-1.4	-2.3	-2.3	-2.8	-3.0	-2.6	-2.7	-2.1	-2.0	-2.4	-3.0	-2.4	-1.4	-1.2
L.baicalensis	1	1	1	1	1	1	1	1	1	1	1	1	1	1		-1.8	-1.8	-2.4	-2.6	-2.1	-2.2	-1.5	-1.4	-2.0	-2.6	-2.0	0.0	1.0
Oncosclera sp.	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1		-2.3	-2.2	-2.4	1.4	-2.1	-2.0	-2.1	-1.8	-2.4	1.7	-1.8	-1.6

Ohridospongilla sp.	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1		-2.8	-3.0	-2.6	-2.7	-2.4	-1.0	-2.2	-3.0	-2.4	-1.8	-1.3
P. globosum	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1		-1.0	-2.5	-0.7	-2.3	-2.6	-1.5	-1.0	-2.4	-2.4	-2.2
P. incrustans	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1		-2.7	-1.2	-2.5	-2.8	-1.9	-1.4	-2.6	-2.6	-2.4
Spongillina sp.	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1		-2.4	-2.3	-2.4	-2.1	-2.7	1.0	-2.1	-2.0
S. vastus	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1		-2.1	-2.4	-1.3	-1.2	-2.2	-2.2	-2.1
S.lacustris	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1		-2.1	-1.9	-2.5	-2.1	-1.5	-1.3
Swartschewskia papyracea	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1		-2.0	-2.8	-2.2	-1.4	-1.2
T. horrida	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1		-1.6	-2.0	-2.0	-1.8
T. pennsylvanica	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1		-2.6	-2.6	-2.4
Echinospongilla brichardi	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	**0.04**	1	1	1	0.16	1	1	1	1	1		-2.0	-1.8
Rezinkovia echinata	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1		1.0
Racekiela ryderii	1	1	0.16	0.16	1	1	1	1	0.16	1	1	1	1	1	0.16	1	1	1	1	1	1	1	1	1	1	1	0.16	

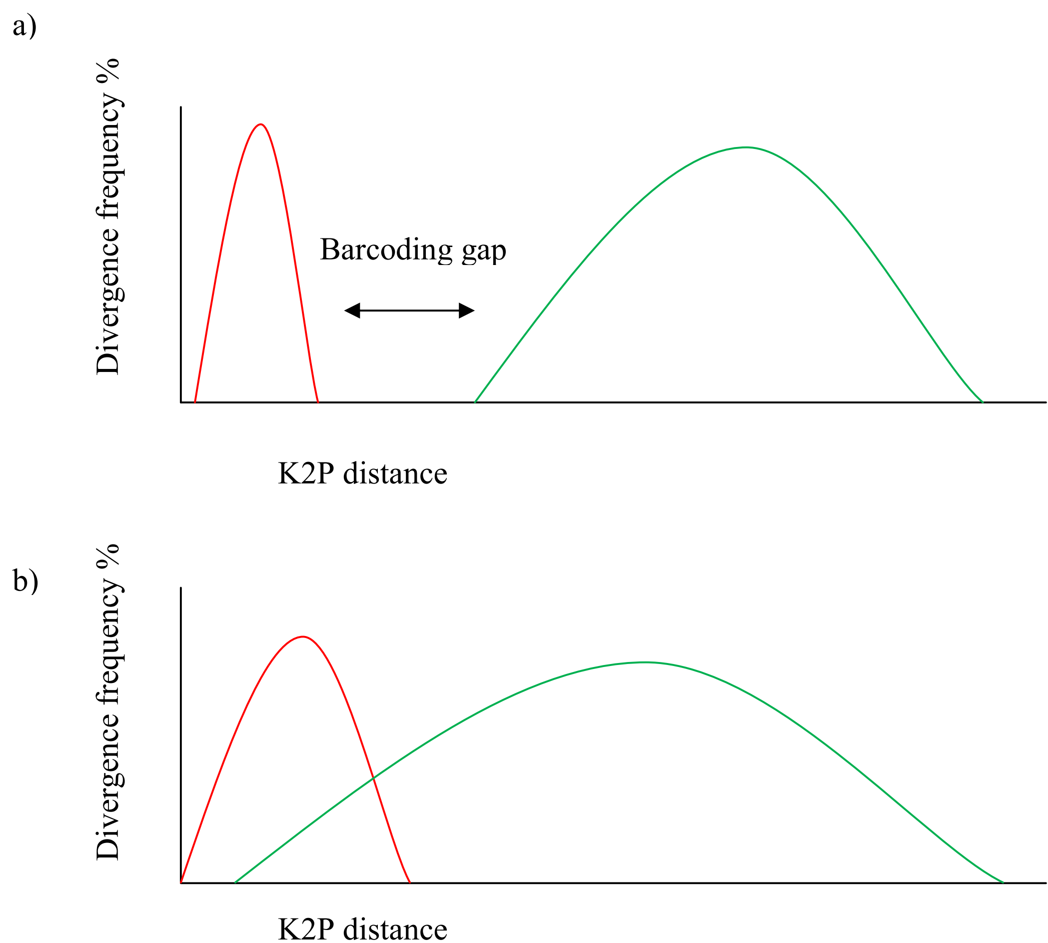


Fig. S1. Diagram to illustrate divergence frequency in intraspecific divergence (red) and interspecific divergence (green) (after Meyer and Paulay, 2005). (a) With a discrete barcoding gap and no overlap. (b) With significant overlap and no gap.

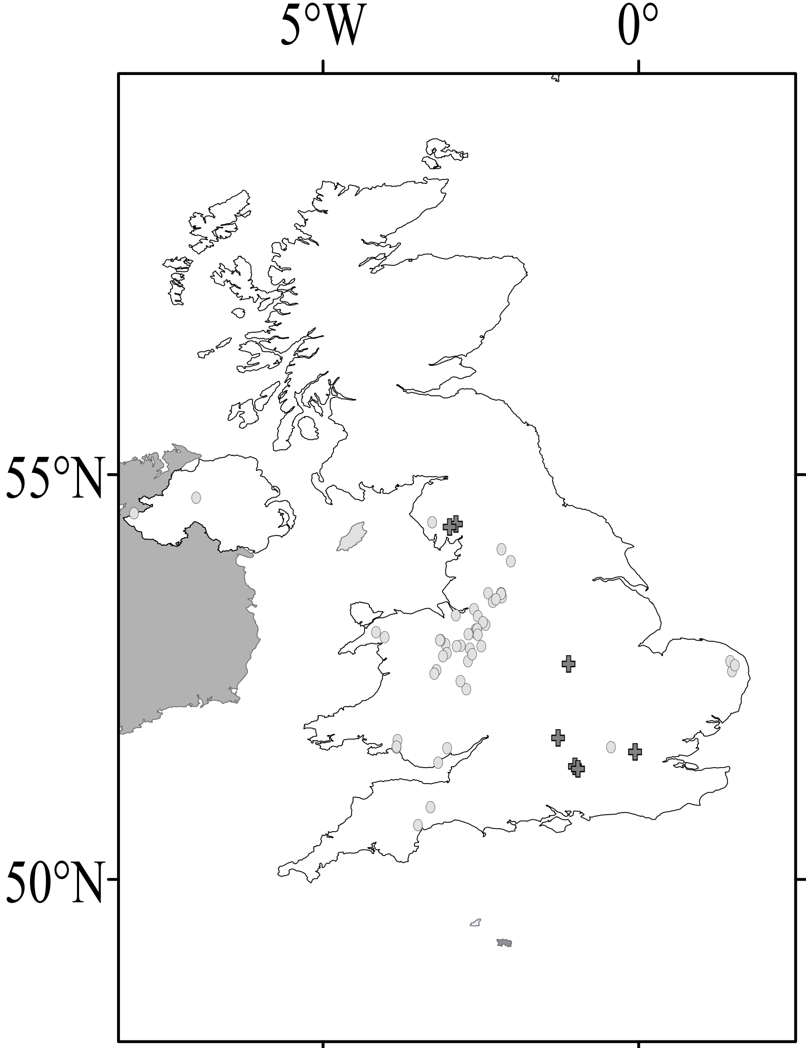
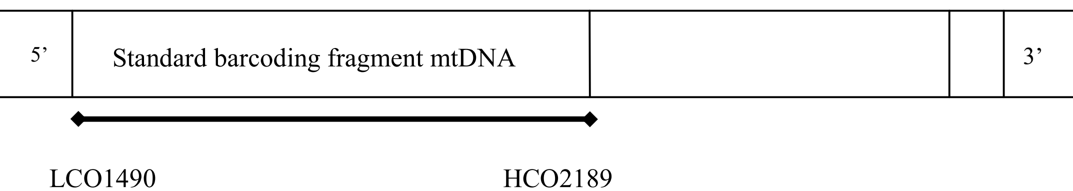


Fig. S2. Distribution of UK sampling sites for freshwater sponges. Data sources: light grey = UK survey sites, dark grey crosses = samples from Natural History Museum.

a)



b)

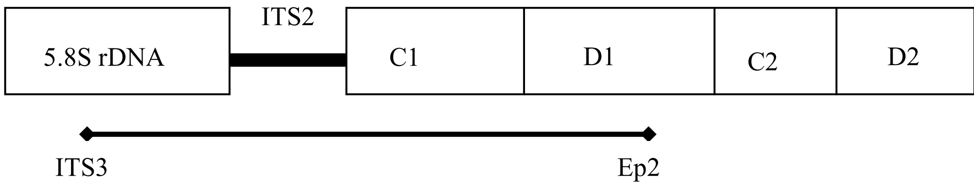


Fig.S3. Primer locations for PCR and sequencing of a) COI gene in respect to marine sponge species *Amphimedon queenslandica* (from position 50 to position 709) and suggested extension (up to position 1297) (Sponge Barcoding Project, www.spongebarcoding.org). b) ITS2 region between 5.8S and 28S rDNA segments after Chombard *et al*. (1998).

Chapter 5: Morphological and genetic analysis of populations of *Spongilla lacustris* Linnaeus 1759 (Porifera: Demospongiae) from UK anthropogenic habitats.

Summary

Traditional sponge species identification depends on morphological features of spicules. However, sessile, aquatic invertebrates, including sponges have been reported to exhibit an adaptive response to variable habitat conditions. This brings into question the usefulness of spicule traits in taxonomy. However, variation in spicule morphology with variations in habitat parameters has not been assessed in UK freshwater sponges. As an alternative to using morphological features, molecular data are widely employed to define species including 28S rDNA and ITS gene sequences. The present study examined populations of *Spongilla lacustris*, a common and widely distributed species abundant in UK canal waterways. Sequences of 28S rDNA genes were used for species identification, and ITS rDNA gene sequences exhibited a single nucleotide polymorphism. Larger megascleres were found at sites with low dissolved phosphorus and increased pH. Water parameters influencing physiological and metabolic processes involved in sponge spicule production may be morphologically important and influence taxonomic criteria.

Introduction

Understanding the mechanisms that produce phenotypic variation is important in evolutionary studies (Conover *et al*., 2006; Conover and Schultz, 1995; Endler, 1977), as an organism's phenotype is the target of natural selection (Roff, 1997). The type of response exhibited by an animal depends on the character and degree of environmental variability (Roughgarden, 1979). A number of basic responses for adjusting phenotypes to environmental variability have been suggested (Mayr, 1963; Levins, 1968), including forming a flexible phenotype modulated by selected features of the environment (plasticity), or genetic variability (Etter, 1988). Significant differences in phenotype across varying environments raise the question of the usefulness of phenotypic traits in taxonomy. Even small differences in phenotype may be important when investigating animals such as sponges that have few defining characteristics used for morphological taxonomy (Klautau *et al*., 1999).

Sessile invertebrates have been shown to exhibit morphological and physiological acclimation with differing environmental conditions (Chappell, 1980; Bell and Barnes, 2000; Fan and Dai, 1999). Marine sponge species exhibit morphological variation in spicule size (Uriz, 1988; Bavestrello *et al*., 1993; Mercurio *et al*., 2000; Palumbi, 1984, 1986) and shape (see Boury-Esnault and Rützler, 1997) in relation to environmental factors. Spicules are part of the skeletal material of sponges (see Bergquist, 1978, Chapter 1) and include megascleres and microscleres. Megascleres are the largest and form the main skeletal framework. Present in some species (e.g. *Spongilla lacustris*), smaller microscleres contribute to the stiffening of the sponge body (Koehl, 1982). Freshwater sponges, and a few marine species, produce gemmules containing specialized gemmuloscleres that are the most useful spicule type for species identification (Penney and Racek, 1968, Chapter 2). However, gemmules may only occur during certain times of the year, and some species (e.g. *S. lacustris*) may produce gemmules without gemmuloscleres (Manconi and Pronzato, 2002). Spicule variation is of particular significance in sponges where morphological features of spicules are used as taxonomic characters (e.g. Hooper and van Soest, 2002; Penney and Racek, 1968). However, the extent of variability in UK freshwater spicule characteristics has not been assessed.

As an alternative to using morphological features, molecular data are widely employed to define species (see Hebert *et al*., 2003; Hebert *et al*., 2004; Radulovici *et al*., 2010; Chapter 4) using a barcoding approach. Analyses of 28S rDNA sequences have been used to identify freshwater sponge species (Addis and Peterson, 2005; Lopp *et al*., 2007, Chapter 2). The internal transcribed spacer 2 (ITS), situated between the 5.8S and 28S rDNA, is also used to identify species and to infer genetic variability among sponge populations (see Addis and Peterson, 2005, Chapter 4). However, while these genetic regions provide taxonomic data, they also require competences in sequencing and bioinformatics (Yoccoz, 2012). In comparison, identification using spicules preparations on light microscopy require less equipment, expense and time. The present study uses both morphological and molecular methods to identify *S. lacustris* from UK habitats, then to reveal variation within this species. The overall goal was to assess significant water parameters that affect morphological variation, contrasting the effects of spicule type and canal ecology.

In some instances the features of the environment that cause sponge morphology to differ are not known. One approach to testing hypotheses about environmental effects on sponge phenotypic variation requires a variable environment. In general, natural freshwater habitats are considered quite variable in terms of physical characteristics and water parameters (Thorpe and Covich, 2009; Pronzato and Manconi, 1994) and anthropogenic systems such as UK canals provide a convenient setting for tests. Here variations in many water parameters e.g. phosphorus, can be attributed to canals passing through different catchment areas (Bishop, 1995; Neal *et al*., 2005; Chapter 3). Additionally, when canal transport was superseded by other transport methods, deterioration of canals resulted in isolated canal stretches with lower anthropogenic input (Willby and Eaton, 1996). These habitat features allow the relative importance of chemical and physical variation in water parameters to be investigated with regard to freshwater sponge populations.

The present study examined populations of *S. lacustris*, a common and widely distributed species (Hooper and van Soest, 2002; Penney and Racek, 1968), abundant in UK canal waterways (see Chapter 2). Initially this study aims to answer the question, is there variation in the size of *S. lacustris* megascleres and microscleres, and then (see Results) how does spicule morphology vary in relation to water parameters in canal habitats? This work uses summer survey data of freshwater sponge colonies, 28S rDNA and ITS gene sequences, *S. lacustris* spicule size, and water parameters to assess factors that determine spicule variability in canal populations. An ANOVA was used to assess spicule variability in megascleres and microscleres. Phylogenetic analysis was used to compare gene sequences from freshwater sponge species. Generalised linear modelling methods (glm) were used to evaluate water parameters, and predict significant parameters for spicule morphology. In this way variation in a defining taxonomic characteristic was related to specific environmental factors.

Methods

Preliminary analysis and sample sites

To ensure that the sites examined encompassed canal stretches with sponge colonies, preliminary surveys of the main canal waterways located in the north-west

of the UK (Fig. 1) were conducted. Twelve shaded sampling sites were identified, separated by >20 km. Sampling sites were located on two replicate waterways, both with isolated stretches of canal, and each site had three sub-sites separated by 0.5 km to 10 km (Fig. 1) with 36 (12x3) sub-sites overall (Supporting Information 5.1, Table S1).

Survey protocol and taxon identification

Twelve key water parameters (see Supporting Information 5.1, Table S2) were recorded monthly at a water depth of 0.4 m at sites, from June to August 2012: temperature, conductivity, total dissolved solids (TDS), salinity, dissolved oxygen, pH, and oxidation-reduction potential (ORP) were measured in situ using a multi-parameter recorder (YSI 560; YSI Inc., Yellow Springs, Ohio, U.S.A.); a 0.1 l water sample was taken for analyses of nitrate, dissolved phosphorus, silicon and calcium following methods of Radojevic and Bashkin (2006); water velocity was measured using a floating ball (see Dodds, 2002). Sub-sites were assessed for sponge presence, and three sub-samples (~1 cm^3) were collected from each sponge colony to a depth of 40 cm. Gemmules were collected with specimens when present. Observations regarding growth form, and presence of gemmules and/or larvae were made *in situ*. Sponge samples were placed in 100% ethanol, and used to identify species.

Taxonomy - Microscopy

To record *S. lacustris*, freshwater sponge species were identified based on spicule morphology following taxonomic keys of Pronzato and Manconi (2001) and Manconi and Pronzato (2002) (for a simplified version of these keys, see Chapter 2). Samples (~1 cm^3) were boiled in 100% nitric acid for 2 min and then left for ~ 24 h, to separate spicules from the tissue. After three washes in water and re-suspension in 100% ethyl alcohol, spicules were transferred to slides. Spicules were examined at 200 times magnification; images were captured with a digital camera and analysed with an image analysis system. The minimum, maximum, and mean (±SE) length and thickness of *S. lacustris* spicules were determined from 20-100 spicules of each type (following Pronzato and Manconi, 2001). Comparisons were made between mean *S. lacustris* spicule values in the present study and those from other research

(Ricciardi and Reiswig, 1993; Barton and Addis, 1997; Penney and Racek, 1968; Pronzato and Manconi, 2001). To assess significant differences in spicule length and thickness between sub-sites, measurements were compared by one-way ANOVA and Bonferroni correction (Zar, 2010), followed by planned contrasts of isolated and connected sub-sites using SPSS v. 22 (IBM Corp, 2013).

Taxonomy – Sequencing

Total genomic DNA was extracted from *S. lacustris* samples using a DNA extraction kit (DNeasy, Qiagen Inc, Hilden, Germany) following the manufacturer's instructions, except that after incubation in lysis buffer the tubes were briefly centrifuged to remove spicules. DNA concentration was quantified using a QuantiT dsDNA assay kit and a Qubit fluorometer (Invitrogen, Carlsbad, CA, USA). The D3 domain of the 28S rDNA together with approximately 150 bp of the 3′ core sequence was amplified using the primers of Lopp *et al*. (2007).

5'-GAC CCG TCT TGA AAC ACG GA-3′

5'-TCG GAG GGA ACC AGC TAC TA-3′

The ITS region situated between the 5.8S and 28S rDNA segments was amplified using the ITS3 and Ep2 primers of Addis and Peterson (2005).

ITS 3:5′-GTCGATGAAGAACGCAGC-3′

Ep2:5′-CTYYGACGTGCCTTTCCAGGT-3′

PCRs were set up in a 20 µl final reaction volume that contained ~10 ng genomic DNA containing the following: 10 µl 2X GoTaq Green (Promega, Southampton, UK), 0.4 pmol of each primer (Eurofins MWG Operon, Germany), 2.0 µl 10X BSA (New England Biolabs Inc., Hitchen, UK). Thermal cycling conditions were: 94 °C for 4 min, followed by 35 cycles at 94°C for 45 s, 45°C for 30 s, 72°C for 45 s, and a final 72°C for 8 min. PCR products were then purified with 0.15 µl shrimp alkaline phosphatase (1000U ml^{-1}) (USB, UK) and 0.03 µl Exonuclease I (20000Uml^{-1}) (New England BioLabs Inc., Hitchen, UK) following the manufacturer's protocol. Sequencing was performed using BigDye v.3.1 chemistry (Applied Biosystems, Life Technologies, Paisley, UK), with ~10 ng of PCR products and 1.6 pmol of primer (forward or reverse) in each reaction. Sequencing products were cleaned by ethanol precipitation, and then separated by capillary electrophoresis on an ABI3130xl. Forward and reverse sequences were checked by eye in Geneious v.6.1.2 (http:www.

geneious.com, Kearse *et al*., 2012) to produce a consensus. Individual sequences were aligned using MEGA v.5 (Tamura *et al*., 2011), and the consensus sequences for each species were then aligned using CLUSTALW v. 2.0.12 (Thompson *et al*., 1994). Sequences were then manually checked and trimmed; alignments were then subsequently revised by eye in an effort to maximize positional homology. Pairwise genetic distances between species were calculated using Kimura's two-parameter (K2P) distance model (Kimura, 1980) using MEGA. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. A distance table was processed to calculate divergence means with and between genera. The sequences were then used for a subsequent Neighbor-Joining analysis (Saitou and Nei, 1987) with a bootstrap replication of 500 replicates (Felsenstein, 1985). Species *Eunapius fragilis* was used as an out-group as this has been shown to form a sister group to *S. lacustris* (Erpenbeck *et al*., 2011).

Statistical analysis

To explore the influence of water parameters on *S. lacustris* spicule characteristics, multiple logistic regression was used in the R 'stats' package (R Core Team, 2014, v 2.12.0). Regressions were performed on the water parameter data set used to record *S. lacustris* spicules. Water parameters and predictor data not meeting the assumptions of normality and equal variances were natural log transformed prior to glm analysis (Zar, 2010) using SPSS. To account for possible collinearity among water parameters recorded at sub-sites, pairwise scatter plots were constructed using R, and covariants that were highly correlated (Pearson product correlation >0.5) were dropped (Zuur *et al*., 2010). In a group of collinear variables the variable that was the most strongly correlated with the dependent variable was retained. The Cook statistic was assessed using R, so that outlier sub-sites with high values (>1) that may dominate glm results (Zuur *et al*., 2010) could be removed. Glm were fitted using Gaussian family type and identity link function to correlate spicule length and water parameters. All possible combination of water parameters were correlated with spicule length. The best models were considered to be those with the fewest predictors and those that received "substantial" support (i.e., ΔAIC <2; Burnham and Anderson, 2002) of the overall best model.

Further analysis was focused on statistically significant parameters (see Results Table 4). A Mann-Whitney U test was conducted to evaluate whether megasclere dimensions varied with a) dissolved phosphorus concentrations and b) pH values using SPSS. Megasclere length was evaluated at (i) high and low dissolved phosphorus values (ii) high and low pH values. High values of dissolved phosphorus concentrations were considered to be >20 μgl^{-1} following eutrophication guidelines for freshwater environments (Radojevic and Bashkin, 2006). High values of pH were considered to be >7.5, the suggested value for eutrophic habitats (Michaud, 1991). Microsclere length variations with ORP values were assessed using a Mann-Whitney U test. Microsclere length was evaluated at average positive or negative ORP values representing oxidising or reducing environments (Dodds, 2002).

Results

Spongilla lacustris *occurrence*

In total 394 sub-samples of 185 sponge colonies collected from 81% of sub-sites were analysed, with sponges absent from remaining sub-sites. A total of 62 *S. lacustris* colonies from 16 sub-sites were identified, which occurred alone or with other sponge species, identified as *Ephydatia mülleri*, *E. fluviatilis*, and *Eu. fragilis.* Although colonies were generally encrusting, at isolated sites, *S. lacustris* occasionally (<10%) occurred in a finger-like growth form (Fig. 2d) with cylindrical branches (up to 10 mm diameter and 10cm high) projecting from an encrusting base. *Spongilla lacustris* colonies were brown or cream, and in some cases (<25%) were bright green (Fig. 2d). Gemmules were brown or white (Fig. 2e). Larvae were rarely (<5%) observed.

Morphological analysis

Spongilla lacustris megascleres were smooth fusiform oxeas with tips from gently to sharply pointed (Fig. 2a). Microscleres were abundant to rare, fusiform oxeas with dense spines distributed along the entire length (Fig. 2b). Gemmuloscleres were contained in all gemmules, and were curved oxeas, covered with large, curved spines (Fig. 2c). Average length of megascleres was 238 µm (±1.4 S.E.), microscleres was 74 µm (±0.8 S.E.), and gemmuloscleres was 66 µm (±2.6 S.E.) (Table 1). Further analysis did not include gemmuloscleres, as

gemmules were not present in all colonies or sub-sites. Average megasclere length was shorter than in studies by Ricciardi and Reiswig (1993) and Barton and Addis (1997). Minimum megasclere length was 48% shorter than data from Penney and Racek (1968). Maximum megasclere length was 22% shorter than data from Barton and Addis (1997) (Table 1). Additionally, there was 67% variation in minimum thickness, and 17% variation in maximum thickness of megascleres (Table 1). Average microsclere length was longer than data from Ricciardi and Reiswig (1993) and Barton and Addis (1997) although thickness data for microscleres were similar to other sources (Table 1).

Analysis of intraspecific variation in spicule size (Supporting Information 5.1, Table S3), indicated a significant difference in both mean length of *S. lacustris* megascleres ($F_{15, 413}$=7.26, p<0.01) and microscleres ($F_{15, 281}$=7.05, p<0.01) between canal sub-sites. Post-hoc planned contrasts (Supporting Information 5.1, Table S4) indicate that megascleres from *S. lacustris* colonies at isolated sites were longer than those from connected sites (p<0.05).

Intraspecific divergence

Morphological identification was complemented with molecular methods, based on genetic divergence. First, consensus gene fragments 301 bp long encoding 28S rRNA sequences data were obtained from colony samples at sub-sites. These sequences were identical and supported the ability of 28S rDNA sequences to identify *S. lacustris* samples (Fig. 3a). The 28S rRNA sequence data also confirmed the identity of three other species (*E. mülleri*, *E. fluviatilis*, and *Eu. fragilis*) and identifications coincided with the morphological identifications. The amplified sequences from species differed in twelve nucleotide positions, and the degree of interspecific divergence, as (i) the base differences per sequence, ranged from 1 to 5, and (ii) the K2P parameter ranged from 0.003% to 0.017% (Table 2a, 3a). This genetic comparison was followed using ITS rDNA sequence data from *S. lacustris* samples (Fig. 3b) and three other species (*E. mülleri*, *E. fluviatilis*, and *Eu. fragilis*). The amplified sequences from four species exhibited differences in the degree of interspecific divergence, as (i) the base differences per sequence, ranged from 35 to 60 and (ii) the K2P parameter ranged from 0.044% to 0.108% (Table 2b, 3b). A total

of 200 *S. lacustris* ITS rDNA sequences represented colony data from each sub-site. There was one single nucleotide polymorphism (A to T, at position 440) found in some colony sequences form sub-sites RCD072 and MGS100, changing one amino acid (threonine to serine) in the translated protein. The Neighbor-Joining analysis illustrates the optimal tree with *S. lacustris* populations and an out group (*Eu. fragilis*) (Fig. 4). The sum of branch lengths was 0.215 and represented the low proportion of sites that were varied.

Habitat characteristics

Data analyses from sites indicated that six water parameters were correlated (Pearson correlation coefficient >0.5): temperature, conductivity, TDS, salinity, dissolved oxygen, and nitrate (Supporting Information 5.1, Table S5). To remove analytical problems associated with collinearity, temperature, conductivity, TDS, dissolved oxygen, and nitrate were removed from the analyses (Zuur *et al*., 2010); this was based on the consideration that conductivity may include charged particles (including those of Na^{+}, Ca^{2+}, and Cl^{-}); dissolved oxygen content is a function of temperature; and nitrate levels are less important than dissolved phosphorus in eutrophication processes (Radojevic and Bashkin, 2006). Megasclere and microsclere length were correlated with thickness (Supporting Information 5.1, Table S6) thus to remove collinearity problems only spicule length was analysed. To determine which parameters may be driving sponge species abundance, all remaining parameters were considered in the initial glm analysis.

Best models with substantial support were considered (Supporting Information 5.1, Table S7), with pH, ORP, and dissolved phosphorus featuring most often. The best model contained either one or two significant parameters (Table 4); specifically, pH and dissolved phosphorus were significant factors for megasclere length models. Larger megascleres were found at sites with low dissolved phosphorus (Fig. 5a) and increased pH (Fig. 5b). A Mann-Whitney U test for independent samples, indicated that megascleres from *S. lacustris* colonies at sub-sites with pH>7.5 are larger (z=3.01, p <0.01) than those at other sub-sites (Fig. 6). With relaxed values of p (p <0.1), ORP was suggested to be marginally significant for microsclere length models (Fig. 5c). However, following this a Mann-Whitney U

test for independent samples, indicated that microscleres were significantly larger ($z=3.85$, $p<0.01$) at sub-sites with average positive values of ORP (Fig. 6).

Discussion

Although Palumbi (1986) considered sponge spicule size variation to be an adaptive response to variable marine conditions, there has been a lack of research in freshwater sponges to investigate similar relationships. The present study was the first to examine morphological variation in populations of *S. lacustris*. With regard to the possible responses to environmental variability (Mayr, 1963; Levins, 1968) *S. lacustris* did not form a constant spicule phenotype at canal sub-sites. Instead megasclere and microsclere variability was exhibited with significant variation in length between sub-sites. However, populations did not differ in 28S rDNA sequences or, in fine scale genetic analysis with ITS sequences. The morphological differences in megasclere characteristics varied with environmental conditions, specifically connectivity, pH and dissolved phosphorus. This brings into question the usefulness of megasclere spicule traits in taxonomy.

In a varying marine environment, the gross morphology of sponge colonies from the same species may differ (Bell and Barnes, 2000). In canal habitats, colony gross morphology for *S. lacustris* varied between encrusting and branching colonies. In contrast to encrusting colonies, forming finger-like extensions may increase the basal to surface area ratio (Bell *et al*., 2002). Although the branching form would appear suited to all environments, it was recorded at isolated sites, possibly where breakages to colony extensions are less likely. An additional feature, colony colour was also variable, with green coloured *S. lacustris* colonies containing zoochlorellae (algal symbionts) (Reiswig *et al*., 2009) occasionally formed. Intraspecific variation in gross morphology and colony colour renders these features of limited use in sponge taxonomy.

In addition to variations in gross morphology and colour, *S. lacustris* showed significant variation in megasclere morphology between canal sub-sites (Supporting Information 5.1, Table S3). The dimensions of *S. lacustris* spicules did not correspond to size ranges recorded in other studies (Table 1) and suggested that

spicule dimensions need to be considered in terms of their environment (Desqueyroux-Faundez, 1990). This is the first study to show how environmental factors are important for a single UK freshwater sponge species. Other studies have documented *S. lacustris* spicule morphology with respect to silicon (Kratz *et al*., 1991; Colby *et al*., 1999) recording thicker megascleres, and longer microscleres in high silicon habitats. In contrast, the present study suggested the importance of pH>7.5, and/or high dissolved phosphorus conditions as drivers of increased megasclere length, and oxidising conditions for increased microsclere length.

It is perhaps not surprising that pH was significant in megasclere length analyses in *S. lacustris* populations, or that ORP was implicated in microsclere length, as pH and ORP are important factors underlying aquatic ecology (Talling, 2010). The behaviour of key cell components (e.g. enzymes) is influenced by both the pH and ORP (Økland and Økland, 1986) and can thus influence metabolic processes in spicule formation. Spicule forming cells (sclerocytes) have abundant mitochondria (Simpson, 1984) and high metabolic activity (Wilkinson and Garrone, 1980) to facilitate the deposition of silica around an axial filament (Simpson, 1984; Yourassowsky and Rasmont, 1983). Silica uptake is affected by sclerocyte metabolism (Maldonado *et al*., 1999), with results suggesting these reactions may have an optimum pH >7.5 in UK populations of *S. lacustris*. Similarly, other freshwater taxa (diatoms) have demonstrated increased silicon uptake at higher pH (9.5) values (Simpson and Volcani, 1981). Significantly shorter megascleres at sub-sites with pH <7.5 may be linked to adverse effects on cell metabolism and a reduction in water filtration reported in low pH environments (Emson, 1966). In habitats with pH <7.5, shorter megascleres may contribute to more compact colonies (Bell *et al*., 2002). Larger microscleres, interspersed with megascleres, in oxidative habitats may mean an increase in structural strength of a colony (Koehl 1982). In both cases, colony structural changes require metabolic energy increases (Reznick and Travis, 1996) for silicon uptake.

Although species occurrence of freshwater sponges has been linked to eutrophic conditions (Økland and Økland, 1996), few studies have examined spicule variability with respect to phosphorus. Canal waterways were located in regions

susceptible to human activities including boat traffic in connected stretches. Influxes of phosphorus (and nitrogen) can occur from urban drainages and/or agricultural land (Neal *et al*., 2005; Carpenter *et al*., 1998; Slater and House, 2001). Canal boat discharge may also contain phosphorus (Eaton *et al*., 2007). Phosphorus is the most common plant-limiting nutrient in inland waters and an increase in concentration can facilitate the growth of phytoplankton, resulting in eutrophication (Dunne and Leopold, 1978; Eaton *et al*., 2007). Large amounts of phytoplankton have been reported to adversely affect filtering in *S. lacustris* (Niven and Steward, 1987). Additionally, algae have been observed to grow over sponges (Sciscioli, 1966), and this may also impact sponge metabolism. Although at 20 μgl^{-1} cut-off of dissolved phosphorus there was no significant difference in megasclere length, the present study demonstrated an overall decline in megasclere length as phosphorus levels increased. Historic low phosphorus content of the local catchment (Willby and Eaton, 2002), and low boat use (Eaton *et al*., 2007) at isolated sub-sites were linked with significantly longer megascleres. Together with water parameters, this could also reflect phenotypic acclimation as in heavily used canals it may be important for *S. lacustris* colonies to produce shorter megascleres forming more rigid sponge tissue (Koehl, 1982).

Although spicule characteristics are diagnostic characters in sponge taxonomy (e.g., Hooper and van Soest, 2002) in the present study, a molecular taxonomic approach using the variable D3 segment of 28S rDNA was also shown to be reliable. The sequence variation of the D3 segment was initially analyzed by Nunn *et al*. (1996) and has been used to identify both marine and freshwater sponge species (Alvarez *et al*., 2000; Lopp *et al*., 2007). Verification of each sequence was important to avoid errors when sponge colonies of different species grow over each other (Reiswig *et al*., 2009), and when sponges are inhabited by a variety of epibionts and/or symbionts (Matteson and Zacobi, 1980; Wilkinson 1978). Distance analysis of consensus 28S sequences for five species indicated low K2P values suggesting low divergence between species (Wörheide *et al*., 2004). K2P values are low compared with other invertebrate studies using this marker (e.g. Zou *et al*., 2012; Zhang *et al*., 2011) and with another sponge study where values of > 0.6% were recorded (Itskovich *et al*., 2013). Although 28S genes were able to distinguish

between different species, additional analysis was needed to potentially identify genetic divergence between *S. lacustris* populations. The ITS region is one of the most variable genes in terms of length and polymorphisms (Coleman, 2003). Intra-specific variability in marine sponge species has been previously observed in the ITS region (Wörheide *et al*., 2004) suggesting its potential use in constructing phylogeographic relationships between populations. However, although extensive intraspecific differences in length of individual spacers (>20 bp) have been observed in other taxa (Wörheide *et al*., 2004), they were not found in *S. lacustris*. The presence of one polymorphic position in ITS sequences may be attributed to mutations or hybridization between species (Wörheide *et al*., 2004), and barely affected the neighbour - joining (NJ) tree. Construction of the NJ tree was based on K2P divergences (e.g. Zhang *et al*., 2011), used when divergence distances are low (Nei & Kumar, 2000) as they are in this study. It is possible that low levels of intraspecific polymorphisms in the ITS region may also be found in other freshwater sponge species and may not be useful for intra specific differentiation as suggested elsewhere (Coleman, 2003). Instead this gene could be used in a similar way to 28S rDNA sequences, for species level identification (Itskovich *et al*., 2013).

If the absence of genetic variability in populations of *S. lacustris* can be confirmed using other sequence data (Itskovich *et al*., 2013), it raises the possibility of asexual sponge dispersal using gemmules. Gemmules were observed more frequently than larvae in *S. lacustris* colonies. Possible dispersal methods for gemmules include; dispersal by waterfowl (Reiswig *et al*., 2009), or dispersal by wind (Bilton *et al*., 2001; Manconi and Pronzato, 2002; Fell, 1989) (Table 5). Once sponges form established populations at a canal site, passive gemmule dispersal mechanisms involving water flow (Reiswig *et al*., 2009) and/or human-mediated dispersal from boat traffic (Panov *et al*., 2004) are possible along connected stretches of canal. Another method of asexual reproduction, sponge fragments, lack the structural characteristics needed for dispersal over distances of more than several km (Reiswig *et al*., 2009) (Table 5). As found elsewhere (Williamson and Williamson, 1978), results suggest larval recruitment does not play a major role in the maintenance of *S. lacustris* populations. Instead, the emphasis on asexual processes involving gemmules maximises the chance of successful dispersal of genetically

similar colonies between connected sites and to isolated canal sites (Bilton *et al*., 2001).

The present study suggests that observed spicule differences in *S. lacustris* reflect responses to different sub-site environments. Compared to the progress made in genetics, there has been relatively little effort to analyse environmental effects on phenotype and their significance (West-Eberhard, 1989). *Spongilla lacustris* is the commonest UK species, as in most areas of the northern hemisphere that have been studied (Økland and Økland, 1996). Spicule size is often used as a distinguish characteristic, however, significant intraspecific differences occurred between *S. lacustris* colonies. For sponges, water parameters influencing physiological and metabolic processes involved in spicule production may be morphologically important. The present study found significant differences in megasclere length in *S. lacustris* driven by environmental pH and dissolved phosphorus levels. Since phenotype is a focus of selection (West-Eberhard, 1989), this also reflects adaptive effects within *S. lacustris* colonies. Palumbi (1986) suggested that variation in spicule morphology derives from a sponge's adaptive response to different environmental conditions e.g. colonies of *Halichondria panicea* from high-wave-force habitats were stronger and less flexible than those from low-wave-force habitats. This study suggests that *S. lacustris* physiological changes occur in spicule size in sponges from oxidative habitats with pH>7.5 and dissolved phosphorus <20 $\mu g l^{-1}$. Further work is warranted on the impact of environmental variables on additional species' acclimation and the usefulness of morphological traits for taxonomy.

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Table 1. Overall variation in *S. lacustris* megasclere, microsclere and gemmulosclere dimensions (n>40) from all sub-sites. Values from the present study are compared with those from Ricciardi and Reiswig (1993), Barton and Addis (1997), Pronzato and Manconi (2001), and Penney and Racek (1968) except when not available (na).

Spicule type		Length (µm)				Thickness (µm)			
		Max	Min	$\bar{x}$	S.E.	Max	Min	$\bar{x}$	S.E.
Megascleres	Present study	317	105	238	1.4	15	4	9	0.3
	Ricciardi and Reiswig	362	158	254	na	17	4	10	na
	Barton and Addis	408	120	287	35	19	2.5	11	2.0
	Pronzato and Manconi	300	90	na	na	18	2	na	na
	Penney and Racek	350	200	na	na	18	6	na	na
Microsclere	Present study	114	23	74	0.8	7	2	3.8	0.1
	Ricciardi and Reiswig	94	32	61	na	8	1	3.5	na
	Barton and Addis	120	35	67	7	7.5	1.5	3.5	0.5
	Pronzato and Manconi	178	25	na	na	8	2	na	na
	Penney and Racek	130	70	na	na	8	2	na	na
Gemmulosclere	Present study	89	29	66	2.6	9	2	5.4	0.3
	Ricciardi and Reiswig	58	18	32	na	7	3	5	na
	Barton and Addis	177	25	89	24	9.5	3.0	4.5	1.5
	Pronzato and Manconi	130	21	na	na	10	1	na	na
	Penney and Racek	130	80	na	na	10	3	na	na

Table 2. Estimates of evolutionary divergence between a) 28S rDNA sequences and b) ITS sequences indicating the number of base differences per sequence between freshwater sponge species.

a)

	Spongilla lacustris	*Eunapius fragilis*	*Ephydatia fluviatilis*
Eunapius fragilis	5		
Ephydatia fluviatilis	3	3	
Ephydatia müllerì	3	3	1

b)

	Spongilla lacustris	*Eunapius fragilis*	*Ephydatia fluviatilis*
Eunapius fragilis	60		
Ephydatia fluviatilis	46	57	
Ephydatia müllerì	39	55	35

Table 3. Estimates of evolutionary divergence between a) 28S rDNA sequences and b) ITS sequences with the number of base substitutions per site from averaging over all sequence pairs between sponge species are shown. Analyses were conducted using the Kimura 2-parameter model (Kimura, 1980).

a)

	Spongilla lacustris	*Eunapius fragilis*	*Ephydatia fluviatilis*
Eunapius fragilis	0.017		
Ephydatia fluviatilis	0.010	0.010	
Ephydatia mülleri	0.010	0.010	0.003

b)

	Spongilla lacustris	*Eunapius fragilis*	*Ephydatia fluviatilis*
Eunapius fragilis	0.108		
Ephydatia fluviatilis	0.087	0.096	
Ephydatia mülleri	0.074	0.093	0.044

Table 4. Glm analyses of megasclere length in *S. lacustris*, indicating final model and significance levels for values of water parameters.

		Estimate	Standard Error	Significance (p)
Megasclere length	Intercept	170.42	29.14	
	ln dissolved phosphorus	-6.18	2.82	< 0.05
	pH	12.42	4.34	< 0.05
Microsclere length	Intercept	75.01	1.78	
	ORP	0.06	0.03	< 0.1

Table 5. Comparison of freshwater sponge dispersal methods indicating stage in life cycle and potential dispersal distance.

	Dispersal mechanism		
Reproductive stage	larvae	fragments	gemmules
Type	sexual	asexual	asexual
Potential dispersal distance	short	medium	short or medium/long
Dispersive agent	cilia and currents	currents	currents (water/wind) or birds or human mediated
Contribution to connectivity among populations	low	medium	medium
References	Fell, 1989; Leys and Degnan, 2001; Reiswig *et al*., 2009	Wulff, 1985, 1991; Reiswig *et al*., 2009	Bilton *et al*., 2001; Fell, 1998; Reiswig *et al*., 2009

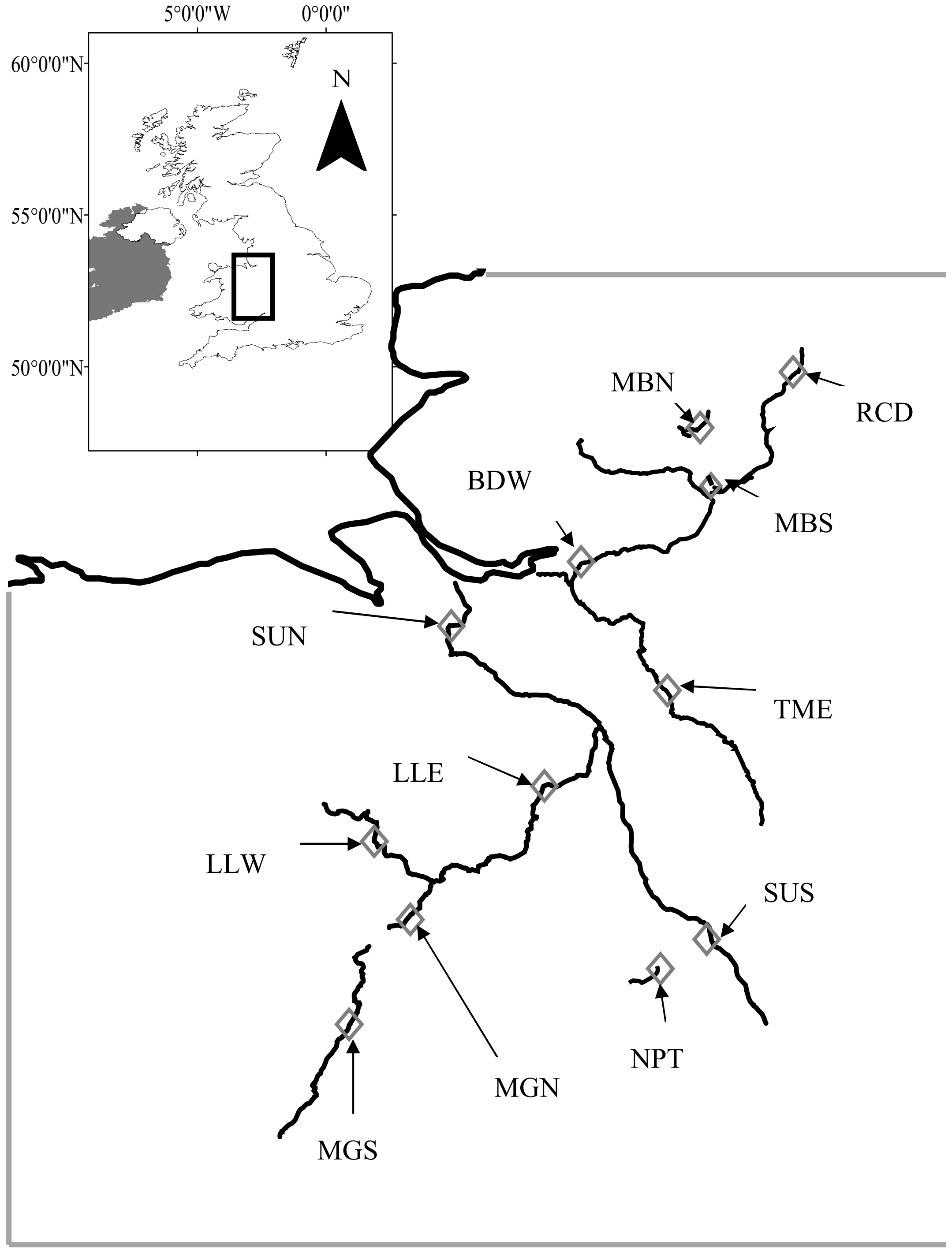


Fig. 1. Approximate location of UK canal sites indicated by site code (Supporting Information 5.1, Table S1 for site names). Only study canals and isolated sections with water are shown.

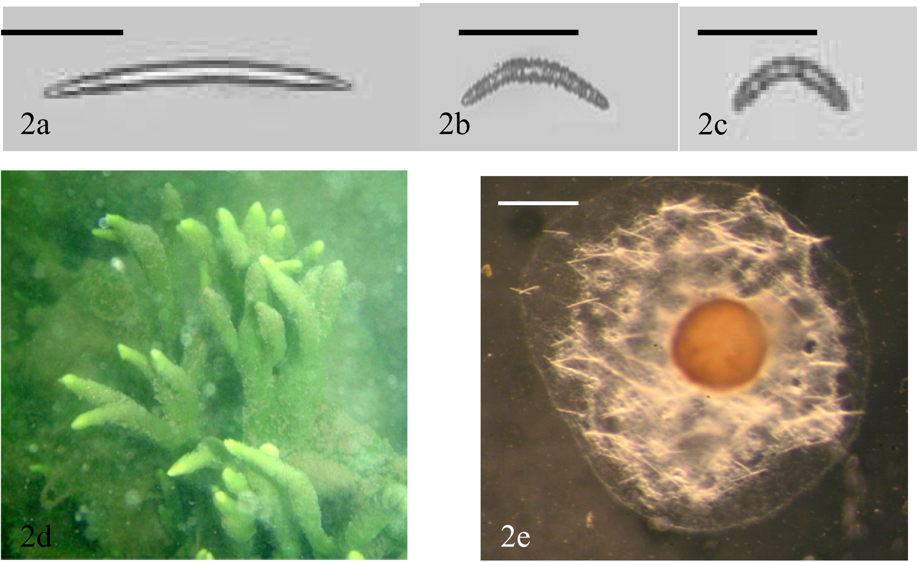


Fig. 2. *Spongilla lacustris* with: a, digital light microscope images of megasclere, and b, microsclere, scale bar = 50 µm, c, gemmulosclere, scale bar = 50 µm, d, green finger-like (approx 10 cm long) colony morphology, e, gemmule and emerging sponge tissue, scale bar = 5 mm.

a)

```
              10         20         30         40         50
     ....|....| ....|....| ....|....| ....|....| ....|....|
28S  CCAAGGAGTG CAACATGCGC GCGAGTCTTT GGGTGAGACG AAAAGCCCTG
              60         70         80         90        100
     ....|....| ....|....| ....|....| ....|....| ....|....|
28S  TGGCGCAATG AAAGTGAAGC GCCGGCTTGC CGGCGCGAGG CGAGAGCCCC
             110        120        130        140        150
     ....|....| ....|....| ....|....| ....|....| ....|....|
28S  TCACGGGTGC CCATCGTCGA CCGATCCTAT TCACTTGTGA AGGGATTCGA
             160        170        180        190        200
     ....|....| ....|....| ....|....| ....|....| ....|....|
28S  GTGAGAGCGT GCCTGTTGCG ACCCGAAAGA TGGTGAACTA TGCCTGAGTA
             210        220        230        240        250
     ....|....| ....|....| ....|....| ....|....| ....|....|
28S  GGGTGAAGCC AGAGGAAACT CTGGTGGAAG CTCGTAGCGA TTCTGACGTG
             260        270        280        290        300
     ....|....| ....|....| ....|....| ....|....| ....|....| .
28S  CAAATCGATC GTCAAACTTG GGTATAGGGG CGAAAGACTA ATCGAACCAT C
```

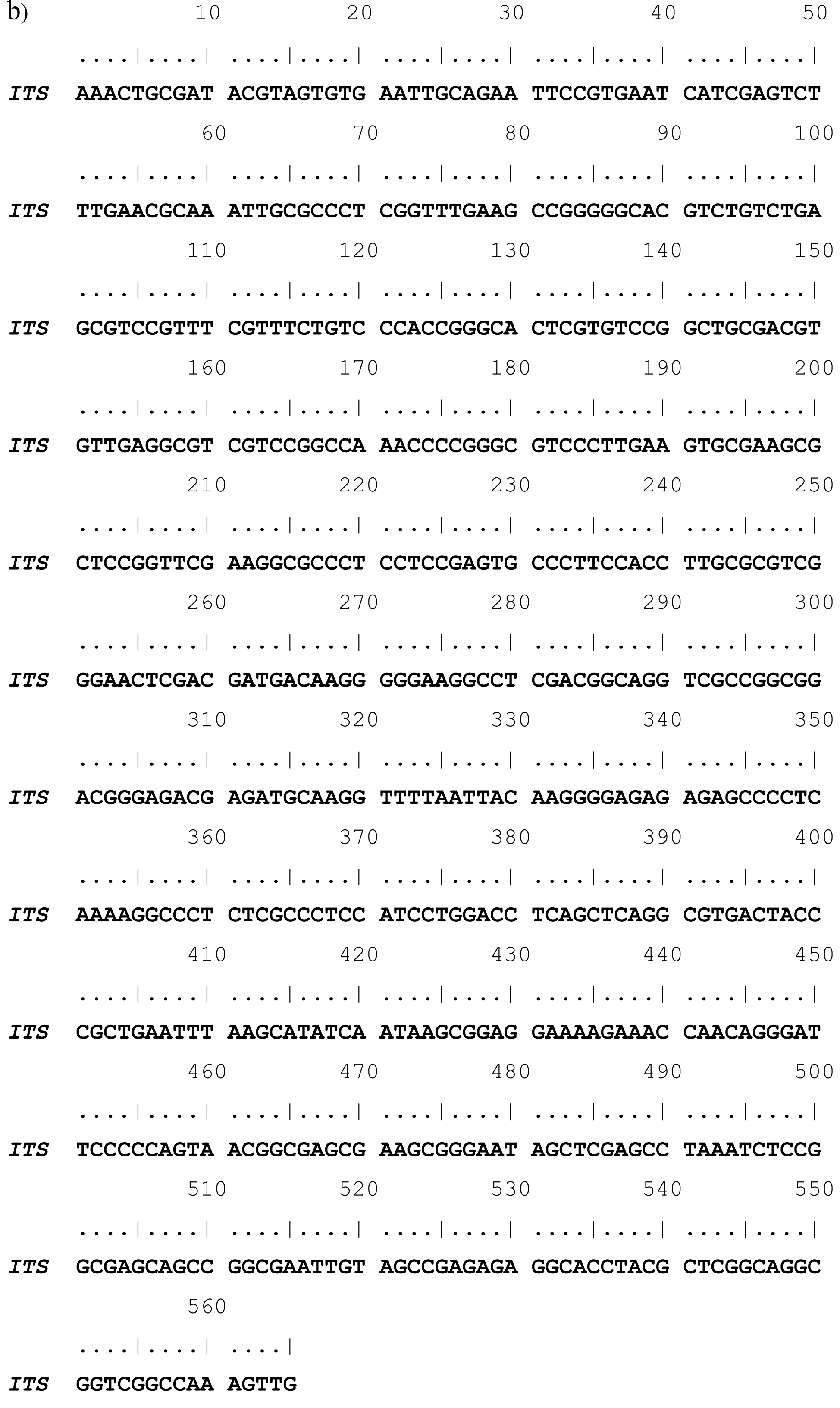


Fig. 3. Sequence from a) the extended 28S rDNA segment and b) the extended ITS2 segments of *S. lacustris*.

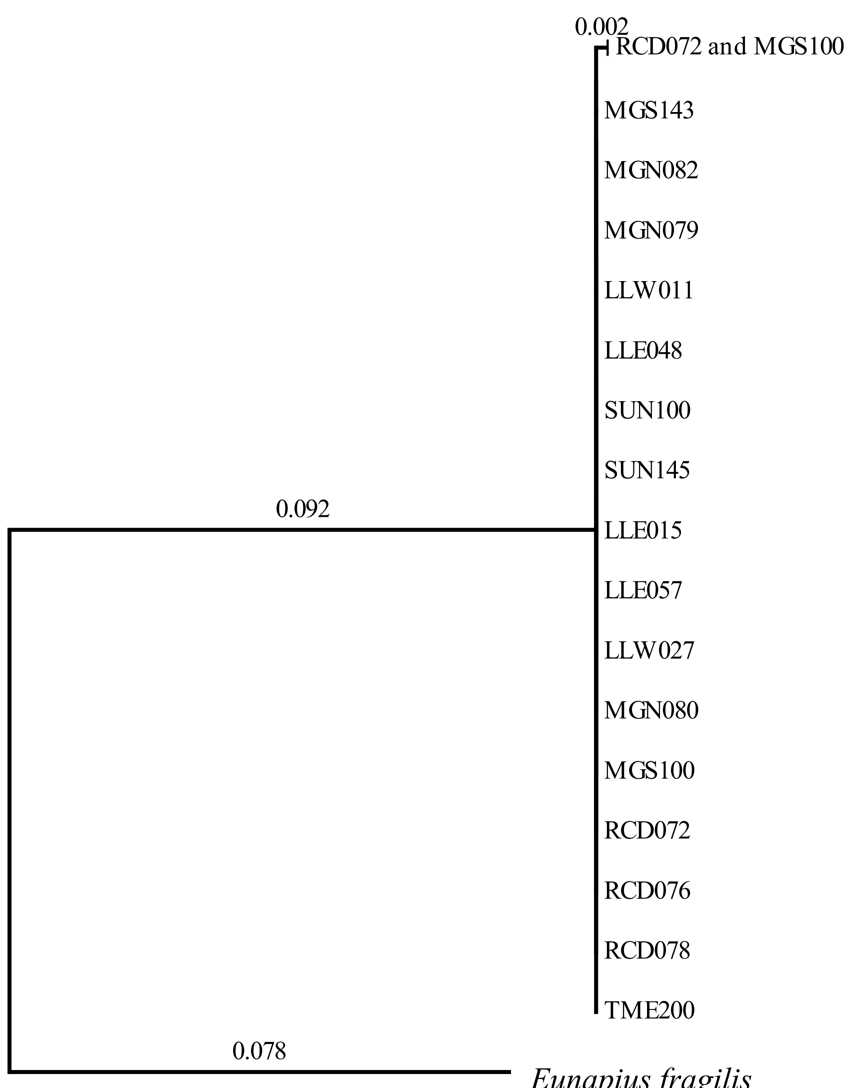
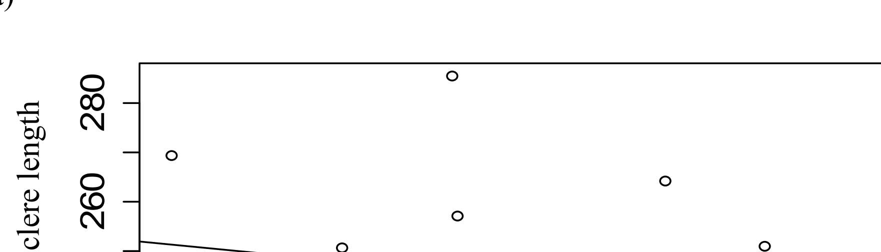
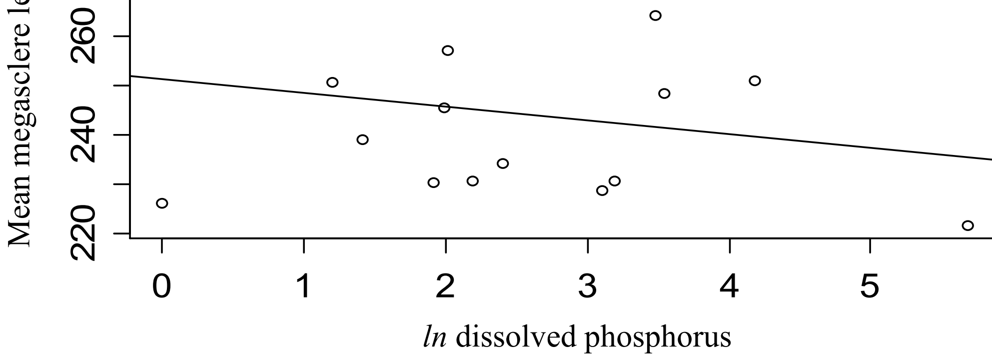


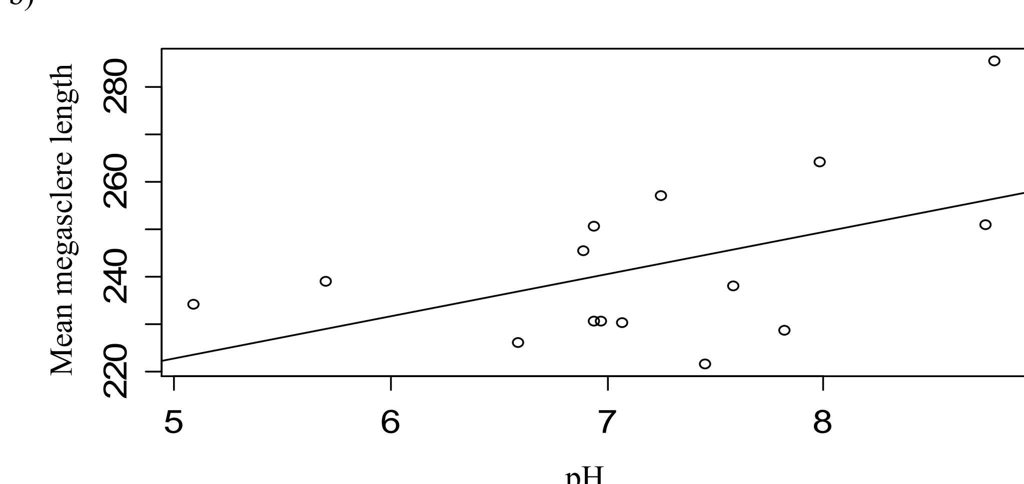
Fig. 4. Evolutionary relationships of *S. lacustris* populations from canal sub-sites and *Eu. fragilis* as an out group using ITS2 rDNA gene. The tree is drawn to scale, with branch lengths (above the branches) in the same units as those of the evolutionary distances used to infer the Neighbor-Joining tree.

a)





b)



c)

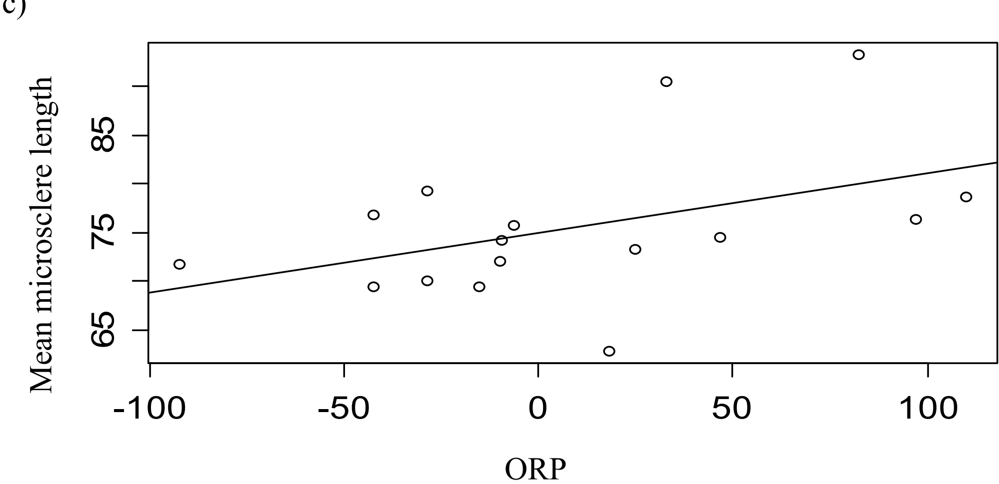


Fig. 5. Glm analyses of correlation of *S. lacustris* a) mean length of megascleres with variations in *ln* dissolved phosphorus, b) mean length of megascleres with variations in pH, and c) mean length of microscleres with variations in ORP.

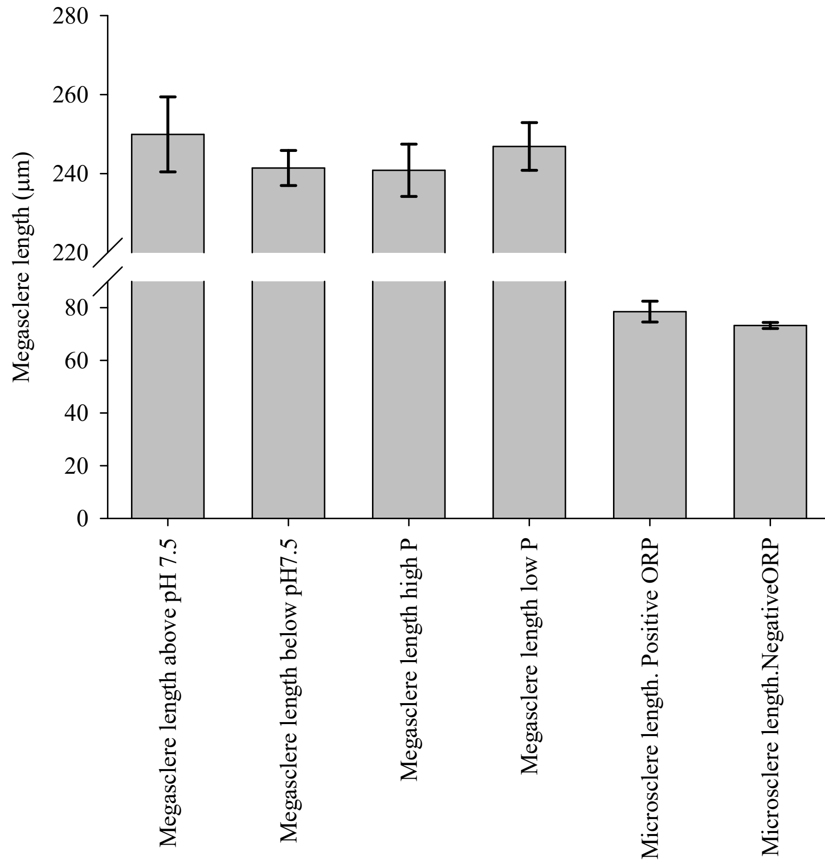


Fig. 6. Mean megasclere dimension at high pH (>7.5) and low pH (<7.5), high phosphorus sub-sites (>20µgl^{-1}) and low phosphorus sub-sites (<20µgl^{-1}), and positive and negative ORP water parameter values. Mann-Whitney U test results indicate a significant increase in length ($z = 3.01$, $p < 0.01$) at sub-sites with average pH >7.5. Megasclere increase in length at low phosphorus sites is not significant using the 20µgl^{-1} cut-off value. Microsclere data show a significant increase in length ($z = 3.85$, $p < 0.01$) at sub-sites with average positive values (oxidising conditions) of ORP. Error bars indicate ±1 standard error.

Supporting Information 5.1.

Table S1. Canal sub-sites with site code. Latitude and longitude also shown.

	Lat	Long	Canal Name	Site code	Site Name
1	52.803	-2.301	Shropshire Union	SUS	Norbury Junction
2	52.887	-2.459	Shropshire Union	SUS	Tyrley Bridge
3	53.043	-2.541	Shropshire Union	SUS	Baddington Bridge
4	53.139	-2.756	Shropshire Union	SUN	Crows Nest Bridge
5	53.172	-2.816	Shropshire Union	SUN	Egg Bridge Waverton
6	53.286	-2.891	Shropshire Union	SUN	Powells Bridge
7	53.069	-2.575	Llangollen Canal	LLW	Wrenbury Heath Bridge
8	52.914	-2.805	Llangollen Canal	LLW	Bettisfield Bridge
9	52.9	-2.876	Llangollen Canal	LLW	Ellesmere Tunnel
10	52.89	-2.99	Llangollen Canal	LLE	Hindford
11	52.93	3.07	Llangollen Canal	LLE	Chirk Tunnel
12	52.97	-3.12	Llangollen Canal	LLE	Plas Ifan
13	52.818	-3.02	Montgomery Canal	MGN	Maesbury Marsh
14	52.817	-3.033	Montgomery Canal	MGN	Gronwyn Bridge
15	52.78	-3.095	Montgomery Canal	MGN	Walls Bridge
16	52.76	-3.09	Montgomery Canal	MGS	Four Crosses
17	52.648	-3.15	Montgomery Canal	MGS	Welshpool
18	52.586	-3.192	Montgomery Canal	MGS	Garthmyl Bridge
19	52.772	-2.38	Newport Canal	NPT	Newport central
20	52.771	-2.384	Newport Canal	NPT	Newport channel
21	52.772	-2.389	Newport Canal	NPT	Tickethouse Lock
22	53.354	-2.632	Bridgewater Canal	BDW	Golborne
23	53.367	-2.579	Bridgewater Canal	BDW	London.Road Grappenhall
24	53.431	-2.312	Bridgewater Canal	BDW	Whites Bridge Sale
25	53.552	-2.169	Rochdale Canal	RCD	Oldham Road Bridge
26	53.527	-2.16	Rochdale Canal	RCD	Chadderton
27	53.509	-2.158	Rochdale Canal	RCD	Failsworth Railway Bridge
28	53.13	-2.374	Trent and Mersey	TME	Wheelock
29	53.2	-2.457	Trent and Mersey	TME	Middlewich
30	53.27	-2.539	Trent and Mersey	TME	Soote Bridge
31	53.48	-2.264	Manchester Bolton Bury	MBS	Middlewood Lock
32	53.479	-2.262	Manchester Bolton Bury	MBS	Margaret Fletcher Lock
33	53.478	-2.259	Manchester Bolton Bury	MBS	River Lock
34	53.554	-2.375	Manchester Bolton Bury	MBN	Bridge 14
35	53.556	-2.378	Manchester Bolton Bury	MBN	Opposite Carlisle Close
36	53.56	-2.387	Manchester Bolton Bury	MBN	Near Hall Lane

Table S2. Significance of water parameter variables recorded in the present study.

Variables recorded at 0.4m	Expected range	Year round range	General significance to sponges - from Harrison (1974) unless indicated.	Significance to *S. lacustris* - from Harrison (1974) unless indicated
Mean water temp (°C)	2 - 20	3.72 - 19.6	May influences sponge life cycle, morphological features or be a limiting factor for growth.	Low temperatures inhibit gemmule development in *S. lacustris*.
Mean Conductivity (µS cm^{-1})	60 - 1200	19 - 910	Index of dissolved inorganic matter. A limiting factor at extremes for some sponge species.	*S. lacustris* and *Eunapius fragilis* occurs more frequently at areas with low conductivity <100 µS/cm (Paduano and Fell, 1997; Økland and Økland, 1996).
Mean water salinity (ppt)	0.03 - 0.6	0.03 - 0.66	Freshwater has salinity <0.5ppt, brackish water has salinity of 0.5 – 30ppt. Euryhaline species may be able to out-compete other species in stretches of canals with higher salinity values (Chapter2).	
Mean pH	6 - 10	5.09 - 9.00	Low values of pH inhibit gemmule hatching.	*Eunapius fragilis* and *S. lacustris* tolerant to wider range of pH. *S. lacustris* occurs in areas with low pH of 6.0 -6.7 (Paduano and Fell, 1997; Økland and Økland, 1996).
Mean dissolved oxygen content (mg l^{-1})	4 – 14.5	4.0 - 15.4	Required for aerobic respiration. Oxygen levels may drop suddenly in areas with pollution.	Oxygen levels may drop in isolated stretches of canals with slow moving water.
Mean value of total dissolved solids in water (TDS) (g l^{-1})	0.1 - 0.9	0.049 - 0.545	Particles of dissolved solids may clog filtering system of sponge especially when growing on vertical canal substrate rather than under rocks. High levels of TDS may prevent development of sponge from a gemmule.	*Eunapius fragilis* is tolerant to higher values of TDS than *S. lacustris*.
Oxidation Reduction Potential	-30 - +100	-196 - 125.5	Measures presence of reducing substances and a water system's capacity to either release or gain electrons in chemical	ORP is an important variable for assessment of invertebrate communities as together with pH it controls

(ORP) (mV).			reactions.	cellular processes (Williams and Fulthorpe, 2003)
Mean Phos-phate conc (P-PO_4) (ppb)	10 - 800	0 - 871.7	Phosphate levels may increase with agricultural run-off. Phosphate influences distributional patterns in some sponge species.	*S. lacustris* less tolerant of eutrophication than *Ephydatia* species (Økland and Økland, 1996).
Mean silicon conc (SiO_2) (ppm)	0.1 - 5	0 - 6.82	Silicon concentration in canal water determined by water runoff in drainage basin and mixing of water from several sources.	Silicon requirement for spicule formation in *S. lacustris*.
Mean calcium conc (Ca^{2+}) (ppm)	5 - 200	6.32 - 110.3	Calcium regulates some aspects of cell metabolism through control of membrane transport processes. Calcium concentration in canal water determined by water runoff in areas with limestone. Levels below 18ppm can limit growth in some sponge species.	Growth of *S. lacustris* not influenced by calcium and this species along with *Eunapius fragilis* has been shown to occur more frequently at areas with low calcium (Paduano and Fell, 1997; Økland and Økland, 1996).
Mean nitrate conc (N-NO_3) (ppm).	1 - 50	0 - 37.29	Nitrate levels may increase with agricultural run-off. Nitrate influences distributional patterns in some sponge species	
Surface water velocity ($m.min^{-1}$)	0 - 10	0 - 12	Oxygen levels and TDS values may be affected by slow moving water. Mild currents transport nutrients High water velocity may prevent gemmule settlement.	Suspension-feeding invertebrates such as *S. lacustris* are influenced by water velocity (Thorp and Covich, 2001).

Table S3. One-way ANOVA results showing significant difference in mean megasclere and microsclere length in *S. lacustris*.

		Degrees of freedom	F statistic	Significance (p)
Megasclere length	Between groups	15	7.26	<0.01
	Within groups	413		
Microsclere length	Between groups	15	7.05	<0.01
	Within groups	281		

Table S4. Following Levene's test and not assuming equal variances, planned contrasts indicated significant difference in megasclere length from colonies at isolated sub-sites.

Levene's test	Contrast	Standard Error	Degrees of freedom	Significance (*p*)
Not assuming equal variances	Isolated	118.38	32.44	<0.05

Table S5. Relationships between water parameters (Supporting Information 5.1. Table S1) recorded at canal sub-sites. Correlation values indicating the proportion of the variance explained by the linear relationship shown in lower left (-ve sign not shown). Significant values (Pearson correlation coefficient >0.5) shown in upper right.

	Wat-er vel	Temp	Cond	TDS	Sal	DO	pH	ORP	N-NO_3	P-PO_4	SiO_2	Ca^{2+}
Wat-er vel		ns	ns	ns	ns	**s**	ns	ns	ns	ns	ns	ns
Temp	0.39		ns	ns	ns	**s**	**s**	ns	ns	ns	ns	ns
Cond	0.25	0.36		**s**	**s**	ns	ns	**s**	ns	**s**	ns	**s**
TDS	0.25	0.32	1.00		**s**	ns	ns	**s**	ns	**s**	ns	**s**
Sal	0.24	0.32	1.00	1.0		ns	ns	**s**	ns	ns	ns	**s**
DO	0.58	0.63	0.47	0.45	0.44		ns	ns	ns	ns	ns	ns
pH	0.49	0.53	0.35	0.35	0.35	0.42		ns	ns	ns	ns	ns
ORP	0.14	0.30	0.67	0.66	0.65	0.19	0.20		ns	ns	ns	**s**
N-NO_3	0.04	0.25	0.47	0.46	0.46	0.00	0.09	0.36		**s**	ns	ns
P-PO_4	0.17	0.30	0.53	0.52	0.52	0.10	0.21	0.30	0.93		ns	ns
SiO_2	0.08	0.40	0.22	0.23	0.22	0.20	0.17	0.02	0.10	0.18		ns
Ca^{2+}	0.27	0.20	0.85	0.85	0.85	0.32	0.40	0.63	0.26	0.35	0.29	

Table S6. Relationships between average spicule dimensions from *S. lacustris* colonies at canal sub-sites. Correlation values indicating the proportion of the variance explained by the linear relationship shown in lower left. Significant values (Pearson correlation coefficient >0.5) shown in bold type in upper right.

Correlation	Megasclere length	Megasclere thickness	Microsclere length	Microsclere thickness
Megasclere length		**s**	ns	ns
Megasclere thickness	**0.65**		ns	ns
Microsclere length	0.39	0.26		**s**
Microsclere thickness	0.30	0.10	**0.63**	

Table S7. Using the best glm for both megasclere and microsclere length, statistically significant water parameters are indicated for models with substantial support using average (mean), or natural log values (*ln*).

	intercept	pH	ORP	*ln* P	Degrees of freedom	AIC	ΔAIC
Megasclere length	170.42	12.42		-6.18	15	135.8	0
	171.46	10.04	0.14		15	136.5	0.7
	184.24	8.4			15	138.8	3.0
Microsclere length	75.01		0.06		15	111.6	0

Chapter 6. Freshwater sponge diversity: why has the amphiatlantic species *Racekiela ryderii* Potts 1882 (Demospongiae: Spongillidae) not been reported from the mainland UK?

Summary

Freshwater sponges are found in many UK habitats but they are difficult to identify, leaving biodiversity estimates are incomplete. Traditional sponge species identification depends on morphological features of spicule types, including megascleres and gemmuloscleres. However, species' delineation depends on sufficient character separation, and the interpretation of sometimes variable traits. The present study examined populations of *Racekiela ryderii*, a rare UK species, with an amphiatlantic distribution, and UK records restricted to the Hebrides. Reflecting megasclere variability a 'form' of *R. ryderii* called *pictovensis* has been described. Following extensive surveys, megasclere morphological variation in *R. ryderii* populations was evaluated, and a difference between the diameters of the gemmulosclere rotules recorded. As an alternative to using morphological features, molecular data are widely employed to define species. Analysis of a partial COI mtDNA gene sequences from *R. ryderii* was conducted, indicating a single nucleotide polymorphism. Although the resolution power of the COI gene was complemented with ITS rDNA sequence data no intraspecific divergence was observed within this species supporting the inclusion of the *pictovensis* form into the species *R. ryderii*.

Introduction

Biodiversity is under increasing threat, with declines in freshwater ecosystems greater than in terrestrial ecosystems (Dudgeon *et al*., 2006). However, knowledge of the total diversity of fresh waters is incomplete especially in non-vertebrates (Dudgeon *et al*., 2006). Freshwater sponges comprise an example of an understudied invertebrate group with taxonomic difficulties (Wörheide and Erpenbeck, 2007). The present knowledge of UK freshwater sponge species comprises six species (see Chapter 2) and, before the present work, was formed from serendipitous and historic research (e.g. Parfitt, 1868; Carter, 1868; Annandale, 1908; Mellanby, 1953). Species of freshwater sponges in the UK (see Chapter 2) comprise: *Spongilla lacustris* (L.), *Eunapius fragilis* (Leidy), *Ephydatia mülleri*

(Lieberkühn), *E. fluviatilis* (L.), *Trochospongilla horrida* (Weltner) and records of *Racekiela ryderii* from the Hebridean Islands. Although rare, *R. ryderii* plays an important role in ecosystem processes (e.g. biofiltration, see Chapter 1) (Poirrier, 1977) and is the most abundant of the five species within *Racekiela* (Bass and Volkmer-Ribeiro, 1998).

Species of *Racekiela* are widely distributed in the eastern parts of North America (Poirrier, 1977). *Racekiela ryderii* is found from the south-eastern United States north to Newfoundland in Canada, with one record from Belize (Potts, 1887; Penney and Racek, 1968; Penney, 1960; Moore, 1953; Poirrier, 1968, 1972, 1977; Gee, 1932). Western European records of *R. ryderii* include Norway (Økland and Økland, 1989), Faroe Islands (Arndt, 1928; Gee, 1932), and Ireland (Gee, 1932; Pronzato and Manconi, 2001; Lucey and Cocchiglia, 2014; Stephens, 1920). Animal and plant species distributed along eastern parts of North America and western parts of Europe are described as having an amphiatlantic distribution (Fig.1). In the UK, *R. ryderii* was recorded from Northern Ireland (Stephens, 1920) and the Hebrides, Scotland (Annandale, 1908; Gee, 1932; Waterston, 1981). However, ecological studies (Økland and Økland, 1989) imply that *R. ryderii* could inhabit many freshwater systems in the UK.

Racekiela ryderii may have been missed at UK sites, and sponge diversity underestimated, due to confusion over morphological features that would enable ready recognition during field surveys. The taxonomy of freshwater sponges has mainly been based on morphological characters, especially form and size of the spicules (Reiswig *et al*., 2009; Penney & Racek, 1968). The presence of gemmules and their morphological traits are diagnostic characters in the current classification at the family, genus and species level (Manconi and Pronzato, 2002). Spicules found in *Racekiela ryderii* include megascleres and it is the only UK species to have gemmuloscleres of two types: birotules and pseudobirotules. Spicule variability in *R. ryderii* populations is a characteristic documented by other research (Potts, 1885, 1887; Stephens, 1912, 1920; Poirrier, 1977; Ricciardi and Reiswig, 1993). Although, Penney (1956) suggested spicule variation was related to habitat conditions, this remains un-quantified. Reflecting megasclere variability a 'form' of *R. ryderii* called

pictovensis was described (Penney and Racek, 1968) differing from the typical form of *ryderii* in the robustness and dense spination of its megascleres. More recently, molecular data have become widely used to define sponge species (Wörheide and Erpenbeck, 2007). However, molecular studies have not included *R. ryderii* type material so there are no reference sequences available. Thus without useful spicule characteristics and the addition of molecular data, species recognition, and the basis of *pictovensis* form of *R. ryderii* in the UK remains unclear.

Another possible reason for the poor recognition of *R. ryderii* was due to taxonomic uncertainty (Wörheide, and Erpenbeck, 2007). Following synonymy with the already occupied genus *Acanthodiscus*, there was a genus transfer to *Racekiela* from *Heteromeyenia* and *Anheteromeyenia* by Volkmer-Ribeiro (1996). Spelling inconsistences are also found as initially both *Racekiela* and *Racekiella* were used (Bass and Volkmer-Ribeiro, 1998). In addition, *Racekiela ryderii* has been spelled *R. ryderi* by researchers including Pronzato and Manconi (2001). The name of this species is *ryderii* in the original description (Potts, 1882) and is used in the present work.

This study provides characters that allow the recognition of *R. ryderii* in the UK, by examining spicule characteristics, and sequences of both COI and ITS genes. Data to support the validity of *pictovensis* form was evaluated. *Racekiela ryderii* samples from Ireland were used for morphological and molecular comparisons. Once means to identify the sponges were established, a systematically study the UK distribution of *R. ryderii* was performed. In order to include as much data as possible, both survey samples and museum samples were included. Altogether, this investigation overcomes the problems associated with a fragmented collection of reports, establishes the extent of *R. ryderii* distribution, and establishes a baseline from which to extend the assessment of this rare but poorly examined species.

Methods

Sponge material – location and data sources

This was part of a systematic rigorous study into freshwater sponges that included surveys of over 200 UK sites. Here, two sources of *R. ryderii* data were

used. First, sponge samples were collected from three upland lakes and colony characteristics were recorded. Sponge specimens were stored in 100% ethanol at 5 °C. Second, a historic search of freshwater sponge specimens from the Natural History Museum, London was undertaken, including samples that may have been mis-identified (see Supporting Information 6.1, Table S1). For all *R. ryderii* samples, the source location was obtained. Samples of *R. ryderii* were obtained from Ireland (Lucey and Cocchiglia, 2014) for morphological and molecular comparison together with records from Stephens (1920) (Fig 2, 3e).

Taxonomy - Microscopy

Samples of *R. ryderii* were identified by light microscopy. First a 1 cm^3 piece of each sample was boiled in 100% nitric acid to separate siliceous spicules from tissue. After three washes in water and re-suspension in 100% ethyl alcohol, spicules were transferred to slides. Spicules were examined at 200 x magnification; images were captured with a digital camera and analysed with an image analysis system. Slides from The Natural History Museum, London, UK were also examined. Characteristics of megascleres, which make up the framework of a sponge skeleton; and both types of gemmuloscleres, which surround the gemmules were recorded. Identification was made using taxonomic keys by Reiswig *et al*. (2009). The minimum, maximum and mean (±SE) length and width of >20 of each type of spicule were determined (following Pronzato and Manconi, 2001). Measurements were compared with Penney and Racek (1968), Ricciardi and Reiswig (1993), Økland and Økland (1989), Stephens (1920), and Manconi and Pronzato (2002). The morphological differences in megascleres that Penney and Racek (1968) used for separating typical *R. ryderii* with *pictovensis* form were recorded.

Taxonomy - Sequencing

Total DNA was extracted from eight sponge samples using a DNA extraction kit (DNeasy, Qiagen Inc, Hilden, Germany). Tissue was processed according to the manufacturer's instructions, except that after incubation in lysis buffer the tubes were briefly centrifuged to remove spicules. DNA concentration was quantified using a QuantiT dsDNA assay kit and a Qubit fluorometer (Invitrogen, Carlsbad, CA, USA).

Polymerase chain reaction (PCR) was used to amplify a 555 bp fragment of the 5′ end of the cytochrome c oxidase subunit I mtDNA gene, using LCO1490/HCO2198 primers of Folmer *et al*. (1994).

LCO1490: 5' - GGT CAA CAA ATC ATA AAG ATA TTG G - 3'

HCO2198: 5' - :TAA ACT TCA GGG TGA CCA AAA AAT CA - 3'

To supplement the COI sequence data the ITS region situated between the 5.8S and 28S rDNA segments was amplified using the ITS3 and Ep2 sponge primers of Addis and Peterson (2005).

ITS3:5′ - GTCGATGAAGAACGCAGC - 3′

Ep2:5′ - CTYYGACGTGCCTTTCCAGGT - 3′

PCRs were set up in a 20 µl final reaction volume that contained ~10 ng genomic DNA containing the following: 10 µl 2X GoTaq Green (Promega, Southampton, UK), 0.4 pmol of each primer (Eurofins MWG Operon, Germany), 2.0 µl 10X BSA (New England Biolabs Inc., Hitchen, UK). Thermal cycling conditions were: 94 °C for 4 min, followed by 35 cycles at 94°C for 45 s, 45°C for 30 s, 72°C for 45 s, and a final 72°C for 8 min. PCR products were then purified with 0.15 µl shrimp alkaline phosphatase (1000U ml^{-1}) (USB, UK) and 0.03 µl Exonuclease I (20000Uml^{-1}) (New England BioLabs Inc., Hitchen, UK) following the manufacturer's protocol. Sequencing was performed using BigDye v.3.1 chemistry (Applied Biosystems, Life Technologies, Paisley, UK), with ~10 ng of PCR products and 1.6 pmol of primer (forward or reverse) in each reaction. Sequencing products were cleaned by ethanol precipitation, and then separated by capillary electrophoresis on an ABI3130xl. Forward and reverse sequences were checked in Geneious v.6.1.2 (http://www.geneious.com, Kearse *et al*., 2012) to produce a consensus. Individual consensus sequences were aligned using CLUSTA LW v.2.0.12 (Thompson *et al*., 1994) through MEGA v.5 (Tamura *et al*., 2011). Sequences were then manually checked and trimmed.

Statistical analysis

For all data, two analyses were used with SPSS v. 22 (IBM Corp, 2013) to assess spicule differences. First, one-way ANOVA with Bonferroni correction (p<0.006, Zar, 2010) was used to test for megasclere differences together with Levene's test examining the assumption of equal variances (Zar, 2010), and Games

and Howell's post hoc test with planned contrasts of sites, adjusted for deviations from homogeneity of variance. Second, the extent of unequal rotule diameters among gemmuloscleres was assessed using a paired t-test with Bonferroni correction (Zar, 2010), with SPSS v. 22 (IBM Corp, 2013).

Results

Gross morphology

Lake colonies of *R. ryderii* were thin hemispherical encrustations (<1 cm thick), with rounded edges with a hard, brittle consistency. Growth was recorded on the top surface of rocks, in large numbers, peaking during July to August and associated with *S. lacustris*. Colonies contained a small number of gemmules.

Spicule morphology

Megascleres varied from straight to slightly curved, amphioxea with rounded or sharply pointed tips resembling amphistrongyla (Fig. 3). Megascleres from Irish samples exhibited branching (Fig. 3e). Megasclere size was variable between sites, exhibiting differences in robustness (Table 1). Megascleres were covered with micro-spines (Fig. 3 a, c) or had sharply pointed spines except at the tips which were smooth (Fig. 3 b, d). Megascleres characteristics typical of the *pictovensis* form with rounded or abruptly pointed tips, were recorded in some samples (Fig. 3c). Other populations were slender fusiform and sharply pointed amphioxea typical for *ryderii*. Maximum megasclere length was longer than recorded in other studies (Table 1). Average megasclere length and width was within the range of most studies, but was longer than the range described by Penney and Racek (1968); and shorter and thinner than outlined in Systema Porifera (Manconi and Pronzato, 2002) (Table 1). One-way ANOVA (Bonferroni corrected) revealed a significant difference in megasclere length across sites ($F_{6,113}$=25.89, p=0.00), and post hoc tests indicated megasclere lengths from sites LLC001 ($\bar{x}$=244.75 µm ± 3.72 S.E.) and NHM050 ($\bar{x}$=262.63 µm ±9.23 S.E.) were significantly longer than those from sites BMT001 ($\bar{x}$=180.34 µm ±4.44 S.E.), NHM030 ($\bar{x}$=171.42 µm ±3.55 S.E.), and LLL001 ($\bar{x}$=163.74 µm ±4.49 S.E.) (Supporting Information 6.1, Table S2).

Although megascleres were most numerous, two distinct classes of gemmuloscleres (birotules and pseudobirotules) were also recorded on sample slides. Birotule gemmuloscleres were shorter on average than pseudobirotules and had flat to slightly umbonate rotules and teeth along the margin (Fig. 4). The shaft was slender or stout, bearing one or a few straight conical spines. The spines were smooth (Fig. 4). A paired-samples t-test indicated a significant difference between the diameter of the smaller rotule ($\bar{x}$=18.54µm ±0.21S.E.) and the larger rotule ($\bar{x}$=21.62µm ±0.35S.E.) of each birotule gemmulosclere (t_{18}=9.53, p =0.00) (Fig. 6). Pseudobirotules were longer with umbonate rotules with re-curved hooks (Fig. 5); shafts had straight or curved spines.

COI and ITS analysis

COI and ITS sequences were obtained from *R. ryderii*, although in Museum samples degraded DNA affected PCR outcomes. A total of 50 sub-samples from 10 sites were sequenced (Supporting Information 6.1, Table S1). Sequencing of COI and/or ITS was successful for sponges at 10 UK sites and 2 sites from Ireland (Fig. 7, 8). Sequences had a conserved primary structure and thus provided additional confirmation of *R. ryderii* species identification. COI sequences were analysed and aligned, then trimmed to a 555 bp sequence. COI sequences obtained from specimens of *R. ryderii* collected at LLL001, NHM140, and NHM030 sites differ by a SNP (single nucleotide polymorphism) (A at position 500) (Fig. 7) to other sequences from the UK and Ireland. ITS sequences were analysed and aligned, then trimmed to a 580 bp sequence including 5.8S rDNA and 28S rDNA sections, although there was no intraspecific divergence between sub-samples from any sites (Fig. 8).

Discussion

As *R. ryderii* has been reportedly common in freshwater habitats of the Irish mainland (at >60 localities, Pronzato and Manconi, 2001; Lucey and Cocchiglia, 2014; Stephens, 1920), it seemed possible it was present on the British mainland. Although, the present detailed study of UK freshwater sponges recorded *R. ryderii* at fewer (13) locations, this represents an increase in range compared to records from

Northern Ireland (Stephens, 1920) and the Hebrides (Annandale, 1908; Waterston and Lyster, 1979; Waterston, 1981). Although not analysed here, a narrow tolerance range of environmental parameters may correspond to the scarcity of *R. ryderii* from UK sites (see Chapter 2). *Racekiela ryderii* has been recorded in habitats with lower maximum values of pH and conductivity than habitats with common European species (*Spongilla lacustris*, *Ephydatia fluviatilis*, *E. mülleri*) (Poirrier, 1969; Økland and Økland, 1989; Ricciardi and Reiswig, 1993), with *R. ryderii* occurring only in non-limestone areas (Stephens, 1920; Harrison, 1974). Additionally, pollution intolerance (Harrison, 1974; Lucey and Cocchiglia, 2014) may correspond to scarcity of *R. ryderii* at some UK canal sites (see Chapter 2), and be a factor in presence at Hebridean sites (Waterstone 1981). More sponge surveys at upland lakes may provide additional locations of *R. ryderii*, with data suggesting that if upland lakes contain *S. lacustris* then *R. ryderii* may also be present (also see Volkmer-Ribeiro and Traveset, 1987; Økland and Økland, 1989).

Figure 1 suggests that European populations of *R. ryderii* occur towards the northern boundary of their range. However, although the UK is at a more northerly latitude band than the contiguous USA, effects from the Gulf Stream ensure areas have a milder climate compared with areas at similar latitudes in eastern USA (Økland and Økland, 1989). Therefore, the climate at which *R. ryderii* occurs in the UK is comparable with conditions at more southerly latitudes in the USA (Økland and Økland, 1989). This distribution of *R. ryderii* is similar to the distribution of perennial grass *Vahlodea atropurpurea* (Wahlenb.) which is also an amphiatlantic species, but absent from central Europe (Haraldsen *et al*., 1991). The present European range of *R. ryderii* may reflect its maximum area. However, similar extensive sampling and examination of Museum samples may uncover the presence of *R. ryderii* in other Western European countries.

The amphiatlantic distribution of *R. ryderii* has been discussed elsewhere (e.g. Lindroth, 1957, 1963; Waterston, 1981) and has been explained in part by three possible paths. First, earlier land connections between UK, other parts of Europe and North America may have formed a wide path in an early phase of continental drift (Stephens, 1912; 1920). The amphiatlantic distribution pattern of *R. ryderii* may

represent population remnants occupying their original areas. This path assumes a slow speciation rate in *R. ryderii*. Although a lack of divergence in ITS sequences in the present study, additional sequence data would be required to estimate speciation rates (see Müller *et al*., 2003). Second, Pleistocene land connections may have formed a path for *R. ryderii* and other species present on North Atlantic islands (Lindroth, 1963; Enckell, 1987; Haraldsen *et al*., 1991). However, although *R. ryderii* is found in north-eastern Canada and the Faroe Islands, it has not been located on the North Atlantic islands of Iceland or Greenland. Third, the possibility of long-distance dispersal of *R. ryderii* gemmules, across the Atlantic Ocean has been proposed (Rees, 1965; Macan, 1974; Lindroth, 1957), with dispersal methods including wind, ocean currents or birds (Stephens, 1920; Økland and Økland, 1989; see Chapter 7). As gemmules do not possess "seed-like" structures to enable them to be windborne (Stephens, 1920), and gemmules would be unlikely to germinate after prolonged immersion in sea-water (Stephens, 1920, Harrison 1974), transport by birds is suggested to be the most likely method. Prevailing winds influencing bird flight across the Atlantic, could account for an amphiatlantic sponge to start in Eastern USA and then disperse to Europe (D. Wilkinson, *pers com*). Dispersal of gemmules by birds has been suggested to be effective both over long distances (see Green and Figuerola, 2005) and in inland waters (Bohonak and Jenkins, 2003). Although transport of gemmules by birds from Eastern USA may have taken place at any time, historic records of *R. ryderii* in Ireland before 1882, (Stephens, 1912, 1920) raise the possibility of *R. ryderii* as a recent UK invader. A dispersal route of *R. ryderii* from Ireland to the Outer Hebrides may be possible as, despite their small size (<2% area of UK), the Outer Hebrides contain a disproportionally large percentage (>15%) of UK freshwater habitats (Waterstone 1981). The occurrence of Hebridean biodiversity studies could also account for the location of *R. ryderii* (see Waterston and Lyster, 1979; Waterstone, 1981).

Spicule morphology can be used to identify freshwater sponge species in the UK. In a species such as *R. ryderii* where megasclere variation has been recorded, the presence and shape of gemmuloscleres is important in distinguishing species (Reiswig *et al*., 2009). Although not in the original species description (Potts, 1882), the significant size difference at each end of birotule gemmulosclere demonstrated in

the present study provides and additional tool for species identification in UK freshwater sponges. This birotule size difference has not been observed in other UK species (see Chapter 2). Before the present study, rotule size differences had only been demonstrated in the genus *Heterorotula* (see Manconi and Pronzato, 2002). In the Australian species, *H. capewelli*, gemmuloscleres similarly have flat rotules with irregularly crenulated margins, and the unequal diameter sizes of rotules range from 24-28 μm and 20-23 μm. These examples of unequal rotule diameter size may be a gemmule adaptation for substrate attachment, as a pavement layer of gemmules attached to the substrate is not present in *R. ryderii* as it is in other UK species e.g. *Eunapius fragilis* (see Chapter 2).

One question addressed by this study concerned the "forms" of *R. ryderii*. Three years after describing the *ryderii* species (Potts, 1882), a sponge collected in Canada was considered distinct and named as species *pictovensis* (Potts, 1885). Subsequently, the typical form of *R. ryderii* was re-defined, adding *pictovensis* as a variety (Table 2). More recently, the variations in "forms" of *R. ryderii* are suggested to be ecomorphs in response to habitat conditions (Poirrier, 1977), and this suggestion is supported by similar forms of *R. ryderii* recorded in Irish, Norwegian, and Canadian habitats (Stephens, 1920; Økland and Økland, 1989; Ricciardi and Reiswig, 1993). Environmental factors have also been suggested to influence length, width and shape of the megascleres in other species (e.g. *S. lacustris* Chapter 5. See also *E. fluviatilis:* Poirrier, 1974; Mysing-Gubala and Poirrier, 1981). The influence of environmental factors in the presence of "forms" of *R. ryderii* is supported by the collection of all the intermediate links between the typical form and *pictovensis* form in a lake (*pictovensis* form), and then tracing it down the course of the stream (typical form) draining the lake (Stephens, 1920).

Both spicule morphology and sequence data can provide information for sponge species identification. Results in this study demonstrated the conservation of *R. ryderii* COI and ITS sequences and low divergence, suggesting there is no genetic basis for "forms" of *R. ryderii* in the genes studied. Although megasclere morphological differences which Penney & Racek (1968) used for separating *R. ryderii* and *pictovensis* form (Fig. 3), were present, all specimens has similar COI

and ITS sequences. Sequence divergence of one base in COI sequences is surprising as identical sequences of this gene are found in other UK freshwater species (see Chapter 4). In comparison, ITS rDNA has been demonstrated to be more variable than COI in sponges (Wörheide *et al*., 2004) and, thus has been used to resolve differences between species (Itskovich *et al*., 2008; Meixner *et al*., 2007) although, in comparison to other sponge species (Wörheide *et al*., 2007; Addis and Peterson, 2005), intraspecific ITS sequence differences were not observed in the present study. These data support the view of Potts (1887), Stephens (1920), and Poirrier (1977) that the *pictovensis* form should be included in the species *R. ryderii*. Meixner *et al*. (2007) proposed a model in which only a few cosmopolitan sponge species (e.g. *Ephydatia fluviatilis*) gave rise to various endemic species. Understanding of the evolutionary events in the process of *R. ryderii* colonisation could be increased by extending the molecular studies to populations in other countries. Further analyses with larger sequence data sets (i.e. additional molecular markers) and by densely sampling populations should be performed in order to gain more conclusive results about *R. ryderii* “forms”.

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Table 1. Size of *Racekiela ryderii* spicule types from the present work and data in North America (Penney and Racek, 1968), Canada (Ricciardi and Reiswig, 1993), Norway (Økland and Økland, 1989), Ireland (Stephens, 1920), and Pennsylvania, U.S.A. (Manconi and Pronzato, 2002).

		Penney and Racek	Riccciardi and Reiswig	Økland and Økland	Stephens	Manconi and Pronzato	This work
Megascleres	Max length(µm)	220	279	315	330	431	352
	Min length(µm)	190	141	165	120	296	160
	Av length(µm)	NA	NA	NA	NA	NA	281
	Max width(µm)	19	21	11	20	26	14
	Min width(µm)	13	1	3	2.5	12	4
	Av width(µm)	NA	NA	NA	NA	NA	8
Birotule gemmuloscleres	Max length(µm)	40	41	NA	35	49	51
	Min length(µm)	30	28	NA	24	33	33
	Av length(µm)	NA	34	27-30	NA	NA	41
	Max width(µm)	4	5	NA	NA	8	5
	Min width(µm)	3	3	NA	NA	5	2
	Av width(µm)	NA	4	2.9-3.9	NA	NA	3
	Av diameter(µm)	25-27	20-28	15-20	20	25-29	19-22
Pseudobirotules	Max length(µm)	75	64	NA	40	92	56
	Min length(µm)	50	46	NA	35	47	36
	Av length(µm)	NA	56	39-52	NA	NA	44
	Max width(µm)	8	8.5	NA	NA	10	5
	Min width(µm)	6	4	NA	NA	5	3
	Av width(µm)	NA	7	3.4-5.1	NA	NA	4
	Av diameter(µm)	NA	17-23	10-12	NA	NA	12-14

Table 2. Megasclere spicule morphology in forms of *R. ryderii* (Ricciardi and Reiswig, 1993; Stephens, 1920) found on both sides of the Atlantic Ocean.

	pictovensis form	Typical *ryderii* form
Habitat	Lakes	Streams and rivers
Gross morphology	Circular, compact, pale yellow colonies that are <60mm in diameter	Circular, dome-shaped, pale yellow colonies
Megasclere length	Slightly curved amphioxea. Short with pointed or blunt ends	Thin, slightly curved amphioxea. Long with pointed ends
Spine density	Dense and abundant spines	Many small spines except at tips
Type of spine	Recurved, strong spines	Procurved, small spines
Length range	120-279 µm	194-330 µm
Width range	1-21 µm	8-13 µm

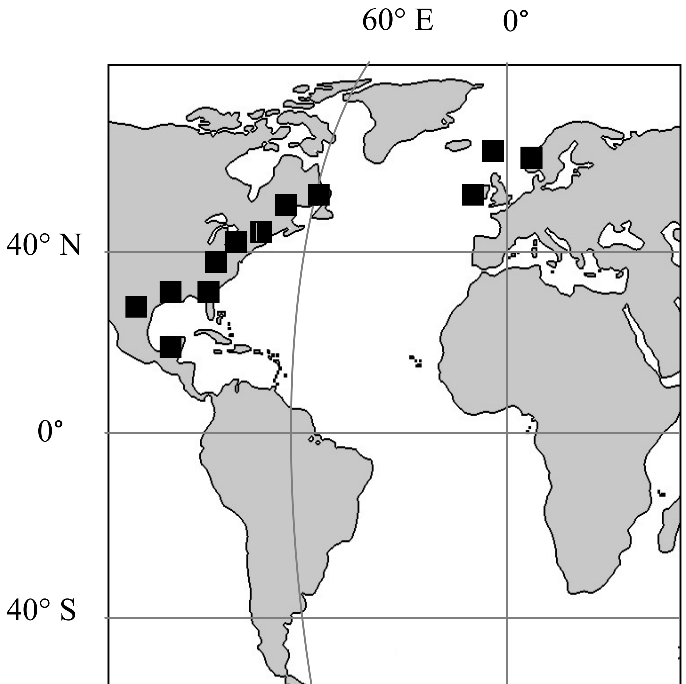

Fig. 1. Globally known distribution of *Racekiela ryderii* outside the UK: Canada (Newfoundland); USA (Texas, Louisiana, Mississippi, Florida, Pennsylvania, Connecticut, Maine); Belize; Ireland; the Faroe Islands; and Norway (Poirrier, 1977; Potts, 1887; Penney and Racek, 1968; Penney, 1960; Moore, 1953; Poirrier, 1968, 1972, 1977; Gee, 1932; Eschleman, 1950; Økland and Økland, 1996; Pronzato and Manconi, 2001; Lucey and Cocchiglia, 2014; Stephens, 1920).

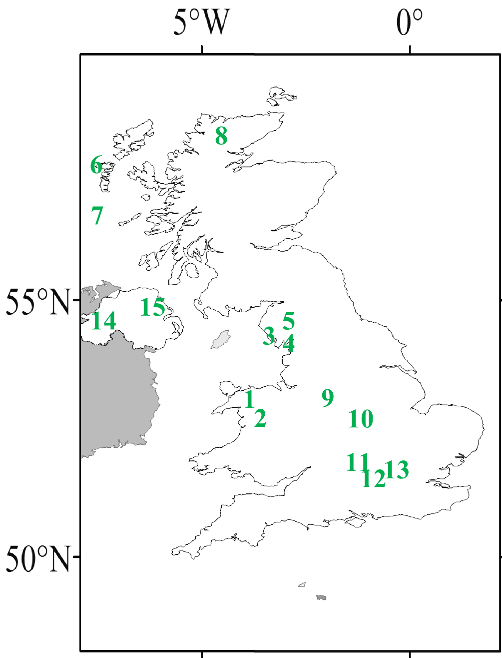

Fig. 2. Known UK distribution of *Racekiela ryderii* with sites represented by 1, 2 (Snowdonia lakes); 3, 4, 5 (Lake District sites); 6, 7 (Hebrides); 8 (Sutherland), 9 (Peak District), 10 (GUC Canal), 11, 12 (Reading sites), 13 (Littleton); 14 (Mallybreen Lough). Data from samples collected in sponge surveys or identified in collections at Natural History Museum, London. Also, additional Northern Ireland sites (15) (from Stephens, 1920).

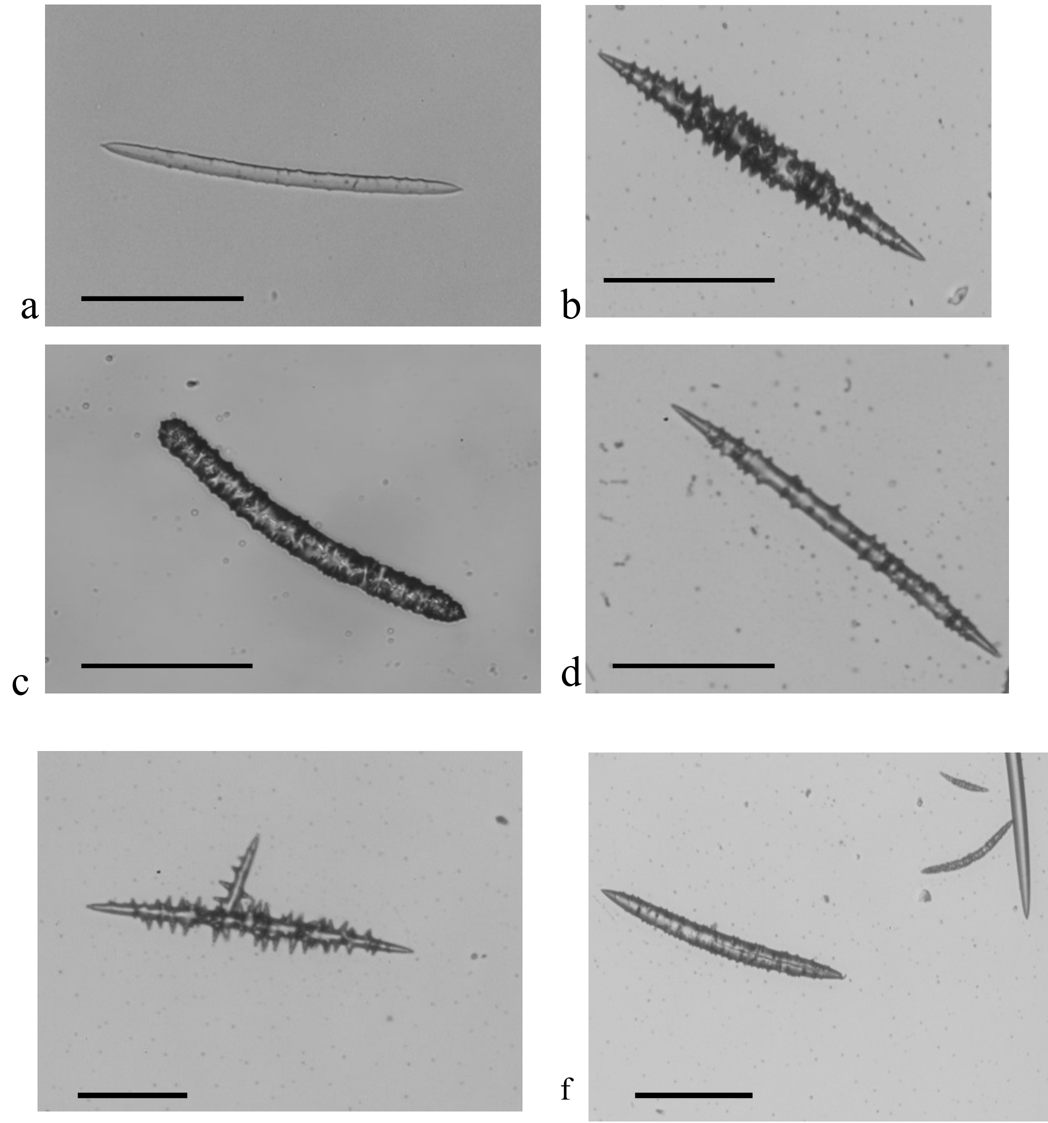

Fig. 3. *Racekiela ryderii* megasclere spicules a) curved, slender, with small spines from the Peak District; b) densely spined, except at the ends from Ireland; c) thicker, curved, with small spines, and slightly blunt from the Lake District; d) straight with spines, except at the ends from Snowdonia; e) samples from sites in Ireland also included branched megascleres; f) spicule mixes with *S.lacustris* (microsclere) also recorded. Scale bars = 100 µm.

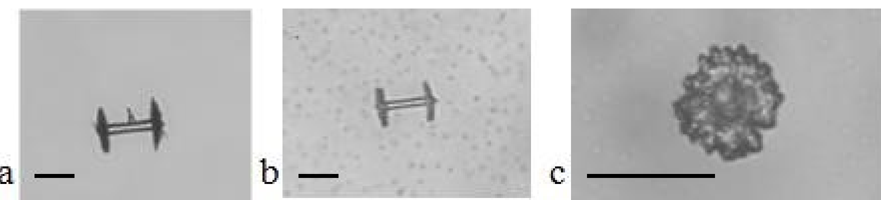

Fig. 4. Rotule gemmuloscleres of *R. ryderii*, a) with one or more spines on a shout shaft and umbonate rotules; b) without spines; c) rotule with numerous small, sub-divided teeth along the margin. Scale bars = 25µm.

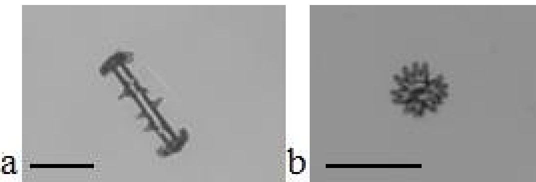

Fig. 5. Pseudorotule gemmuloscleres of *R. ryderii*, a) curved spines on shafts and curved hooks on rotules; b) rotules with deeply incised teeth. Scale bars = 25µm.

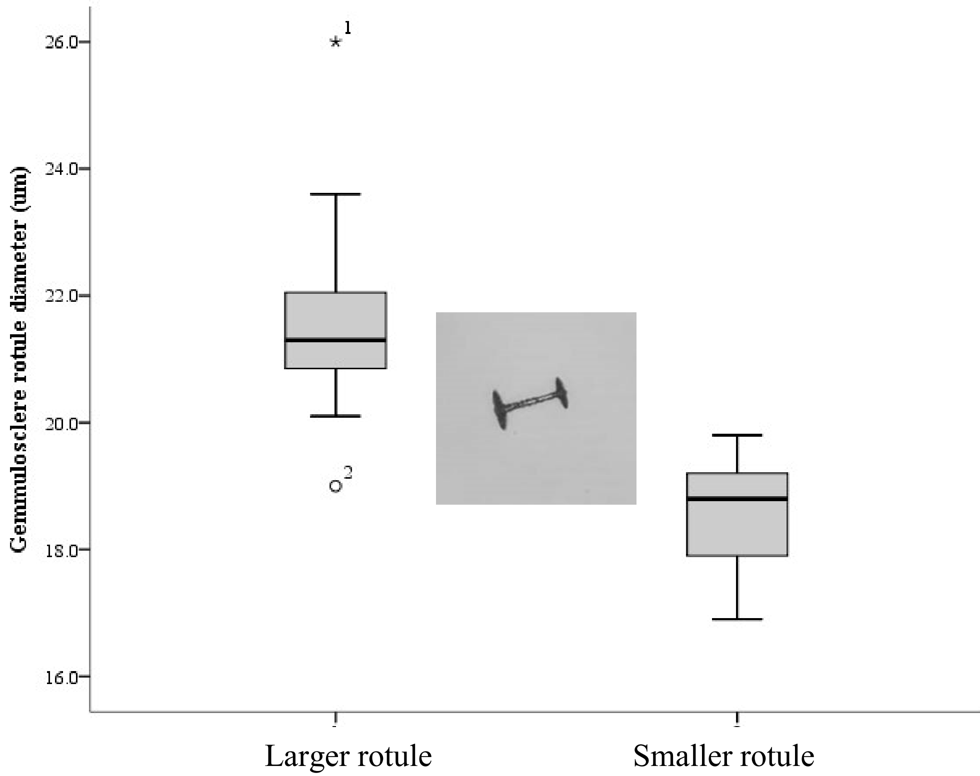

Fig. 6. Variation in rotule diameter at each end of rotule gemmulosclere (see inset image). Median values are included; the rectangles contain values between the first and third quartiles; the bars connect the extreme values; outliers are indicated.

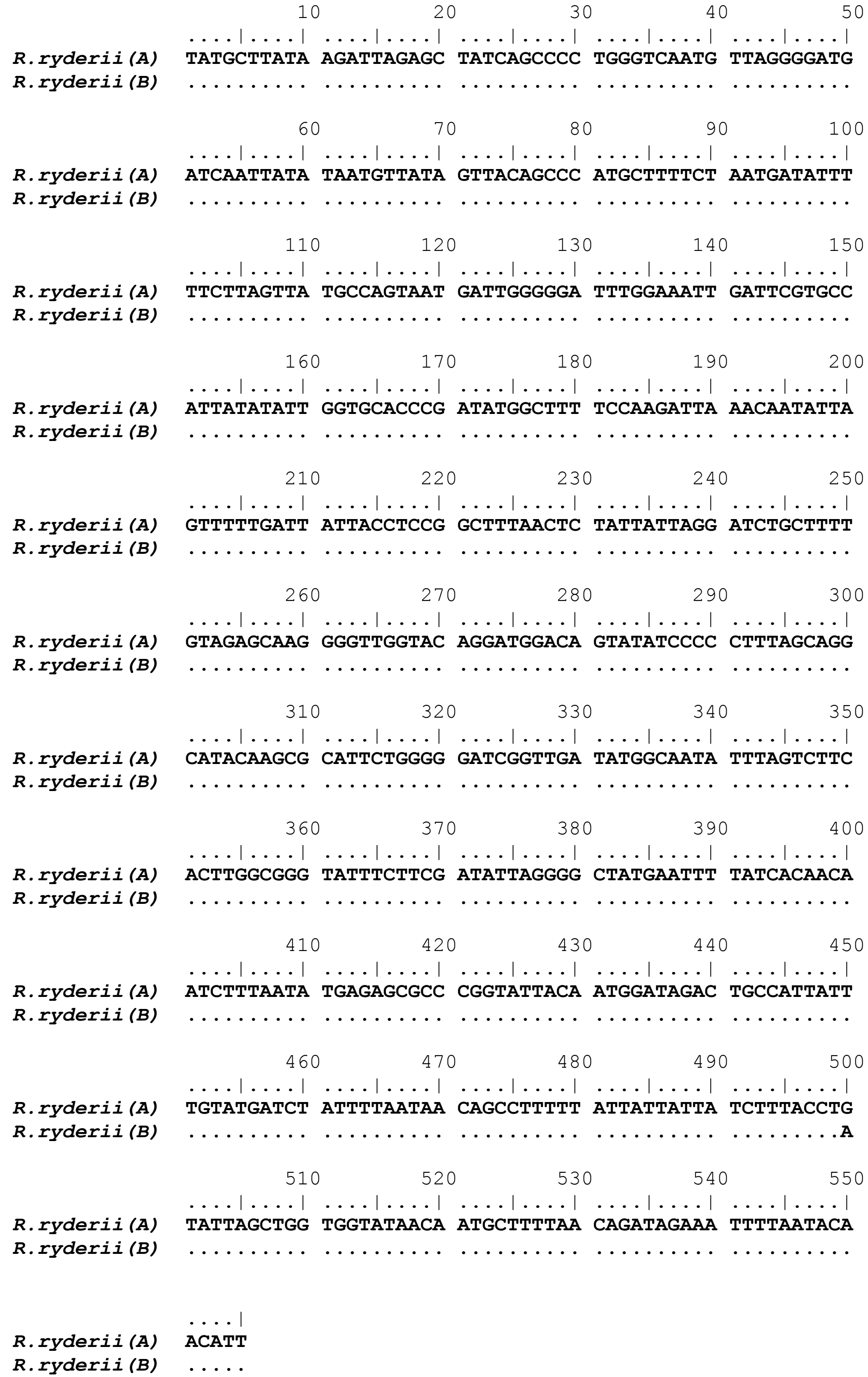

Fig.7. The extended COI rDNA domain from *R. ryderii* indicating one base difference. Identities have been indicated by dots and gaps.

Fig.8. The extended ITS rDNA domain from *R. ryderii*.

Supporting Information 6.1.

Table S1. UK data sources with habitat type. Latitude and longitude also shown. Additional Northern Ireland data from Stephens (1920).

	Lat	Long	Habitat	Site Code	Site Name	Data collected
1	53.015	-4.016	Lake	LLL001	Llyn Llagi	Sponge sample, environmental data
2	53.072	-4.151	Lake	LLC001	Llyn Cwellyn	Sponge sample
3	54.430	-3.262	Lake	BMT001	Burnmoor Tarn	Sponge sample
4	54.372	-2.991	Lake	NHM140	Priest's Pot, Lake District	Sponge sample
5	54.363	-2.986	Lake	NHM570	Esthwaite, Lake District	Slide
6	56.983	-7.464	Lake	NHM240	Barra, Hebrides	Slide
7	57.317	-7.321	Lake	NHM230	Grogarry, South Uist	Slide
8	58.571	-4.739	Lake	NHM590	Sutherland	Slide
9	53.221	-1.687	River	NHM050	Peak District	Sponge sample
10	52.686	-1.102	Canal	NHM070	GUC Leicester Line	Sponge sample
11	51.380	-0.954	River	NHM030	Swallowfield, Reading	Sponge sample
12	51.418	-0.999	Lake	NHM110	Searles Farm, Reading	Sponge sample
13	51.417	-0.460	Reservoir	NHM100	Littleton Reservoir	Sponge sample
14	54.543	-7.989	Lake	MAL001	Mallybreen Lough	Sponge sample

Table S2. Megasclere size data from *R. ryderii* populations obtained from samples collected in sponge surveys or identified in collections at Natural History Museum, London.

Site Name	Mean megasclere length (µm)	Standard Error (S.E.)	Minimum length (µm)	Maximum length (µm)
Llyn Llagi, Snowdonia,Wales	163.74	4.49	125.40	226.70
Burnmoor Tarn, Lake District, England	180.34	4.44	127.50	260.50
Llyn Cwellyn, Snowdonia, Wales	244.75	3.72	237.31	248.71
Swallowfield, Reading, England	171.42	3.55	153.10	201.10
Priest's Pot, Lake District, England	215.20	17.51	165.20	247.00
Peak District, England	262.63	9.23	150.10	307.90
GUC Leicester Line, England	176.28	31.04	121.60	240.10

Chapter 7. General Discussion

General Introduction

Biodiversity is often expressed as species richness or diversity (Ehrlich and Ehrlich, 1992) and provides a foundation for ecosystem studies (Hebert *et al*., 2003a). The present study investigated patterns of freshwater invertebrate diversity and assessed mechanisms of diversity by targeting UK freshwater sponge assemblages. Despite the importance of sponges in aquatic ecosystems (Reiswig *et al.,* 2009), and their value as model organisms (Rivera *et al.,* 2010), relatively little was known about patterns of freshwater sponge species diversity, or the mechanisms underpinning their diversity (NBDC, 2010). This research has attempted to overcome these problems with a comprehensive study of this faunal group. Specifically, at the outset of this thesis, five key questions were devised which informed a series of testable hypotheses, and data from this study have been applied to draw conclusions on freshwater sponge diversity in the UK.

Research areas

Five key questions addressed in this thesis:

i. To compile a species list, identify species distribution, and describe the freshwater sponges which occur in the UK as without knowing the current distributions of these native invertebrates it is not possible to identify changes in community composition over time (Chapter 2). This involved species identification using morphological methods and supported by a molecular approach, based on sequencing a section of 28S rDNA.

ii. To provide fundamental knowledge to facilitate the use of freshwater sponges in ecological studies and determine factors influencing freshwater sponge species presence/absence and abundance in canal habitats (Chapters 2 and 3). The overall goal was to provide summer demarcation values of important water parameters that affect sponge abundance in canal habitats, contrasting the effects of seasonal parameter variability, species requirements, and canal ecology.

iii. To identifying freshwater sponges using spicule morphology and COI barcoding from freshwater species, and compare with marine sequence data (Chapter 4). The reliability of species identification using spicule morphology was assessed. The study then quantified COI sequence divergence between pairs of species freshwater

sponges collected in UK habitats. A similar genetic evaluation of COI sequence divergence in marine sponge sequences from GenBank was included to examine intra- and interspecific variabilities for sponge diversity.

iv. To analyse the mechanisms of diversity of a common UK freshwater sponge population (*S. lacustris*) using morphological methods and specific genetic characteristics (Chapter 5). The overall goal of this study was to assess variation in the size of *S. lacustris* megascleres and microscleres, and then relate how spicule morphology varied in relation to water parameters in canal habitats. The degree of population genetic variability (28S rDNA and ITS genes) was investigated.

v. To analyse the mechanisms of diversity of a rare UK freshwater sponge population (*R. ryderii*) (Chapter 6). This study provided characters that allow the recognition of *R. ryderii* in the UK, by examining spicule characteristics, and sequences of both COI and ITS genes. The overall goal was to assess *R. ryderii* distribution, and establish a morphological and genetic baseline from which to extend the assessment of this species.

Using sponges to assess freshwater biodiversity

To address the first of these key questions rigorous sampling of over 200 sites over six years confirmed the identity of five UK species of freshwater sponges (see Pronzato and Manconi, 2001): *Ephydatia fluviatilis*, *E. mülleri*, *Eunapius fragilis*, *Racekiela ryderii*, and *Spongilla lacustris*. Additionally, another species, *Trochospongilla horrida*, was recorded from one site. These data provide the first formal record to quantify UK freshwater sponge species diversity. Although *E. fluviatilis*, and *S. lacustris* have previously been recorded in the UK, *R. ryderii* was thought to be restricted to the Hebrides and *T. horrida* has never before been reported in the UK. Species distribution patterns differed, with some species common and widespread (*S. lacustris*, *E. fluviatilis*) while others were patchy (*E. mülleri*, *Eu. fragilis*) or rare (*R. ryderii*, *T. horrida*). It is possible that the uncommon species *R. ryderii* has a wider distribution than indicated by this research as it was found in some upland lakes, habitats that are relatively inaccessible compared to streams or canals, so serendipitous finds are less likely. It is possible that the uncommon species *T. horrida* has a wider distribution in south western England as this was not the most heavily sampled area although has been sampled historically (Parfitt, 1868;

Carter, 1868). With its distinct gemmule characteristics, *T. horrida* can be distinguished from other species with spined megascleres, and now it has been recorded in the UK it may be found in other faunal surveys.

These six UK species comprise a third of all described species in Europe, demonstrating that freshwater sponges are more diverse and widespread in the UK than previous records would indicate. Unlike previous research (e.g. Gugel, 2001), samples were from natural freshwater habitats (lakes, rivers, streams) and anthropogenic habitats (canals), taken throughout the year, and from all four countries in the UK. Additionally, the use of 28S rDNA sequence data together with analysis of morphological features allowed conclusive species identification. The present rigorous survey assisting the clarification of sponge diversity patterns in UK inland waters, and demonstrated for the first time in the UK, that species coexisted. These, and other data (Harrison, 1974; Poirrier, 1969), suggest that coexisting species may not be in direct competition for the same food, instead, are able to utilise all types of available particles (Francis and Poirrier, 1986). Considering sampling effort in the present study, and from species assessment in other European countries (see Chapter 1, Table 2), it is unlikely that additional sampling will increase species richness. Instead future efforts should be directed towards refining species distribution data.

Now that freshwater sponge biodiversity has been determined then an additional area of interest for future investigations could include the possibility of investigating sponge species for bioactive molecules. Sponges (as well as associated microbes) exhibit a variety of unusual chemical constituents (Reiswig *et al.,* 2009), with marine sponge species producing an array of bioactive molecules used to treat human diseases (Sipkema *et al.*, 2005). However, although the prospect of discovering bioactive molecules in freshwater sponges such as those in marine species has been suggested (Reiswig *et al.,* 2009), it remains untested. Freshwater sponges have been used since the 18th century, in powdered form ("Badiaga") to rub on the torso of patients with lung diseases or on affected areas of patients with rheumatism (Sipkema *et al.*, 2005). This powdered sponge mix consists of several freshwater sponges including those also found in the UK (*S. lacustris*, *E. fluviatilis*,

E. mülleri, and *Eu. fragilis*) (Sipkema *et al*., 2005). Unlike many marine species, these freshwater species can be cultured from gemmules in the lab (see Chapter 1 Fig. 1 f, g) and, thus, may be more convenient organisms to screen for medically active molecules.

To address the second key question, sponge abundance was recorded together with twelve targeted canal water parameters (Chapter 3) to assess the drivers of species diversity. An important aspect of the analyses was dealing with collinearity (correlation between parameters), which could have increased type II errors (Zuur *et al*., 2010). Previously, calcium was considered to be the main factors determining sponge presence (Jewell, 1939). However, calcium is one component that can potentially influence conductivity measures (Radojevic and Bashkin, 2006). In the present research, unlike other investigations (e.g. Gugel, 2001; Lucey and Cocchiglia, 2014) all ionic contributions to conductivity and total dissolved solids (TDS) data were considered. Dropping correlated parameters avoided confusing statistical analyses where nothing is significant, and instead clarified key parameters (Zuur *et al*., 2010) with species demarcation values. In this way it was concluded that *S. lacustris* and *E. mülleri* show significantly higher abundances at summer salinity levels <0.20 ppt, and *Ephydatia fluviatilis* and *Eu. fragilis* show significantly higher abundances at summer silicon >0.70 mgl^{-1}. That only two spong species were linked with silicon concentrations suggested a requirement for materials to synthesise larger spicules and may also suggest a degree of complexity in sponge particle digestion. Overabundant particles can be quickly cycled through the sponge, while other food particles (such as those with silicon), present in lower concentrations in the feeding suspension, are held within the sponge for longer times so that they can be encorporated into archaeocytes (Frost, 1980) (Chapter 1). Sponges, therefore, are selective feeders, not in their initial uptake of particles but in their ultimate use of these resources (Reiswig *et al*., 2009).

This is the first time that demarcation levels have been assessed as previously, drivers of sponge species diversity had largely been restricted to tolerance levels based on historic data (see Harrison, 1974), with ranges for UK sponge species given for between 8 to 24 parameters. Investigations into drivers of

sponge species diversity were not laboratory based manipulative experiments, but instead investigations relied on ecological surveys and statistical approaches to form conclusions. Consequently, the presence of outlier results was observed and conclusions were focussed at habitat level. More work is needed to find out why different species are affected by specific parameters, and an alternative to an ecological survey would be to create large-scale experiments (see Belas *et al*., 1989) to test each water variable (e.g. silica) and its effect on sponge population abundance.

One factor in the presence of outlier results was large abundances of some *S. lacustris* colonies. The present work has demonstrated that this *S. lacustris* is the fastest growing UK sponge species in freshwater habitats. Space is important for sessile animals such as sponges, so large colonies dominating all the available substrate prevents other species co-existing. Within a suitable habitat, sponge growth is controlled by environmental features such as a hard substrate. Only a few species, (*S. lacustris* and, to a lesser extent, *E. mülleri*), have been recorded growing in soft sediments (Reiswig *et al*., 2009). In addition, sponges are excluded from regions with high flow (e.g. canal weir) due to physical disruption; and regions with wave action or ice scour (Reiswig *et al*., 2009).

Parameter collinearity and the presence of outlier results were two issues that arose during the investigation. Other considerations that arose during the planning of the investigation included the targeting specific parameters to be recorded, and not merely measuring convenient parameters based on the features of a hand-held multimeter. The present study recorded more variables than in other similar investigations (e.g. Dröscher and Waringer, 2007, Gugel, 2001) as what parameters are recorded can be due to cost rather than the major drivers of ecosystem functioning. In addition, the present study ensured there were the same number of sub-site replicates at a site, all were below a canal bridge, and measurements were taken on vertical substrate. Pseudo-replication was avoided by having independent sub-sites, and not pooling data from sub-sites before analysis (Krebs, 1989). These considerations are in contrast to other surveys (e.g. Dröscher and Waringer, 2007, Gugel, 2001) where it is unclear as to why specific sites were visited. Also, by clearly defined sampling sites as those with environmentally different canal

conditions, increased independence of observations, reduced experimental bias (Krebs, 1989), and promoted interpretation of differences in sponge diversity in terms of differences in water parameters.

These strategies to address diversity data analysis in order to avoid errors are particularly relevant when results have the potential to be used to guide management decisions, to ensure that the best policies are derived from ecological studies. Very few studies have directly investigated how the managed and artificial character of anthropogenic waterways affects faunal diversity. However, with increasing deterioration of natural freshwater habitats, investigating anthropogenic environments is becoming more important (Pinheiro *et al*., 2015). There will be differences in environmental stress between study sites. However, sections of canals that become isolated following a canal breach create potential for conservation of diverse taxa (Willby and Eaton, 1996). More surveys of canals in other parts of the UK could be carried out as they are relatively easy to access compared to some natural freshwater habitats, and run through urban areas where other freshwater habitats may be limited. Over 250 million people visit canals *per annum* showing appreciation for 'living' waterways (British Waterways 2007; IWAAC 1996). Sponges have key biological functions, these are part of ecosystem functioning and therefore impact ecosystem services. Thus diversity studies on widespread and ecologically important sponges have the potential to enhance public perception of anthropogenic waterways especially since this study has demonstrated they can grow to cover sizable areas, and as sessile invertebrates, cannot escape scrutiny.

An overall goal in the present study, as well as in ecology is a better understanding of patterns of diversity (Baber *et al*., 2004). As sponge data for this investigation (and for Chapter 2) were recorded an additional aspect of canal faunal diversity was observed i.e. the presence of zebra mussels (*Dreissena polymorpha*, Pallas 1771). This raises the possibility of future investigations to assess the effectiveness of freshwater sponges in the control this invasive species. Zebra mussels are considered to be one of the most important invasive freshwater species in the world because of their economic and ecological impacts (MacIsaac and Grigorovich, 1997). Originally native of the Caspian and Black Sea regions

(Karatayev *et al*., 2003), zebra mussels recently (c 1980) invaded Western Europe and have spread rapidly and widely across the UK (Aldridge *et al*., 2004). The development of the British canal system facilitated zebra mussel dispersal since increasing levels of connectivity in freshwater systems can transport invasive species (A Baker *pers comms*). Zebra mussels were often observed growing on vertical canal substrate during this study, with numbers have been increasing in many habitats forming dense "mats" (Aldridge *et al*., 2004). Freshwater sponges have been observed growing on and over zebra mussels (*S. lacustris* and *E. fluviatilis* in Chapter 2; also Gugel, 2001) (Fig. 1a) resulting in a detrimental effect in zebra mussel populations (Ricciardi *et al*., 1995). The spreading of a sponge across any surface involves the overgrowth of microscopic algae and bacteria, although this has not been investigated in detail. Although, sponges can encrust on aquatic macrophytes, without harming them, growth over bivalves blocks siphons and thus impacts feeding mechanisms. Potentially freshwater sponges may have a similar effect on a more recent invasive species, the quagga mussel *Dreissena bugensis* (Aldridge *et al*., 2004; www.bbc.co.uk/ news/science-environment), although, fortunately, these were not observed during the present research. Investigations in relation to control of invasive species by freshwater sponge colonies should take the highest priority for further studies.

Genetic characteristics of freshwater sponges

Previously, just as the diversity patterns of UK freshwater sponge species had been overlooked, so too had their genetic characteristics (Reiswig *et al*., 2009). This is in contrast to genetic studies with marine sponges (e.g. Erpenbeck *et al*., 2002; Lavrov *et al*., 2005; Wörheide, 2006; Uriz and Turon, 2012). The study of genetics in sponges has a dual application it can be used to help taxonomic studies and understand phylogenetic relationships. To address the third key question the variability of COI in freshwater and marine sponges was investigated. The use of genetic markers to aid sponge taxonomy began in the early as 1990's (e.g. Kelly-Borges *et al*., 1991; Thorpe and Solé-Cava, 1994). Following these early developments attempts to standardise the protocols for species determination have included the use of COI barcoding. Results and meta-analysis demonstrated the conservation of freshwater sponge COI sequences and low divergence in both

freshwater and marine sponges. There were lower levels of intra- and interspecific sequence divergence within freshwater species, genera and families than in marine sponges. Even using a more variable marker did not indicate sequence diversgence. Basal metazoans need a combined approach including careful sequence data on diagnostic SNPs rather than % divergence, sequence data from mitochondrial and nuclear genes, and assessment of morphological data.

Although, mitochondrial DNA sequences are important in sponge phylogenetic studies, only a restricted subset of mitochondrial genes has so far been assessed and more research on other genes, including those from the nuclear genome, is necessary (Uriz and Turon, 2012). As well as diagnostic SNPs in COI sequences of freshwater sponges, non-coding regions of mtDNA may provide additional genetic information. Other research (Pleše *et al*., 2011) has demonstrated that freshwater sponges possess large non-coding regions in comparison to mitochondrial genomes of other taxa and comparative analysis of these regions may provide a higher degree of divergence than in COI sequences together with phylogenetic information. Other research (Erpenbeck *et al*., 2009) suggests that repetitive elements in non-coding regions indicate either an early origin or multiple independent invasions from seawater to freshwater in the evolutionary history of sponges. The three or four suggested separate invasions were then used to classify freshwater sponges into families (Reiswig *et al*., 2009). However, more mitochondrial genome data from sponges are necessary for insight into evolutionary rates of non-coding regions, to provide insights to evolutionary history events.

As well as mitochondrial DNA sequences, the D3 expansion segment of 28S rDNA, and internal transcribed spacer regions (ITS) were used to assess sponge biodiversity (Chapters 2, 5, 6). In each case diagnostic SNPs provided information for species differentiation but not intraspecific divergence. Other possible genetic analyses of sponge diversity include the use of amplified fragment length polymorphisms (AFLPs) in combination with other markers at sites where species co-exist (Gigliarelli *et al*., 2008). Also, microsatellites which are small DNA stretches consisting of a repeated core sequence of a few base pairs can be used for sponge studies of population differentiation, and genetic diversity (Uriz and Turon,

2012). Both AFLPs and microsatellites occasionally have been used in marine sponge studies, but only once in freshwater sponge studies to distinguish between two *Ephydatia* species (Gigliarelli *et al*., 2008; Uriz and Turon, 2012). Although they have the potential for future use, the time-consuming effort and costs required to develop them *de novo* for each target species made their use impractical in this investigation. Using next generation sequencing methods (Riesgo *et al*., 2012, Uriz and Turon, 2012) may allow faster and more efficient development of microsatellites for freshwater sponge population studies.

Population studies

To address the fourth key question, detailed mechanisms of diversity were assessed using *S. lacustris* populations. Although, adaptive spicule size variation has been demonstrated in marine sponges (Palumbi, 1986), there has been a lack of similar research in freshwater sponges. The present study has resulted in demarcation values with significantly longer skeletal spicules at pH >7.5, and positive values (oxidising conditions) of oxidation reduction potential (ORP); and an overall decline in megasclere length as phosphorus levels increased >20$\mu g.l^{-1}$. Although, pH, and ORP (and phosphorus to some extent) are the main factors underlying all aquatic ecology (Talling, 2010), few studies have examined them with respect to spicule variability. Shorter megascleres in *S. lacustris* forming more rigid sponge tissue (Koehl, 1982) could reflect phenotypic acclimation in heavily used canals with increasing phosphorus levels.

The degree of *S. lacustris* population genetic variability (28S rDNA and ITS genes) was investigated. However, although extensive intraspecific differences in length of individual spacers (>20 bp) have been observed in other taxa (Wörheide *et al*., 2004), they were not found in *S. lacustris*. The degree of genetic similarity (28S rDNA and ITS gene sequences) in *S. lacustris* population suggests future investigations using sponges as a model organism to investigate dispersal in sessile invertebrates. The initial challenge for any sessile invertebrate species to inhabit a site is getting to it. Freshwater sites are surrounded by semi-dry lands, drainage and other barriers. As sponge populations were located in a variety of natural and anthropogenic habitats then this implies some ability to disperse.

The distribution of sponges in the UK has been explained in part by dispersal of gemmules (Chapter 5). Such dispersal mechanisms might include the ability of gemmules to withstand desiccation over long periods during which they may be transferred, and the increase in spiculation of the gemmule wall, decreasing the likelihood of physical damage. Gemmule characteristics include production in large numbers, reduced metabolic rates, and ability to survive for one day out of water (Simpson and Fell, 1974, Pennak, 1989; Gugel, 2001). These characteristics allow gemmules to be passively dispersed by wind blowing them away from pieces of drying macrophyte (Freeland *et al*., 2000; Manconi and Pronzato, 2002; Fell, 1989). Wind generated transport processes resulted in frequent long-distance dispersal of seeds from trees (Nathan *et al*., 2003). Gemmules, which are often smaller than tree seeds, therefore also might move considerable distances. In addition small hooks and spines on gemmuloscleres affect attachment to feathers of water birds that migrate at the same time of year as gemmules are produced (Simpson, 1984; Reiswig *et al*., 2009; Ricciardi and Reiswig, 1993; Freeland *et al*., 2000; Manconi and Pronzato, 2002; Fell, 1989). The wide distribution of *S. lacustris* (Chapter 2) suggests it may have developed successful gemmule characteristics to increase dispersal likelihood. Similarly, gemmulosclere rotules of cosmopolitan species, *E. fluviatilis*, have incised margins and hooks on the shafts (Chapter 2) with the potential to increase the likelihood of attachment to birds. The present study suggests that *E. fluviatilis* is the most successful UK species in dispersing to new habitats as this species has a wide UK distribution (Chapter 2); it was located in a variety of habitats (Chapter 2); and displayed phylogenetic relationships with species located in ancient lakes, cave sponges, and UK species (*E. mülleri*, *R. ryderii*) (Chapter 4). In comparison, gemmuloscleres of *T. horrida* have smooth margins and may be less able to attach to birds, possibly contributing to its scarcity.

Methods of dispersal units between water habitats by birds first suggested by Darwin (1859) also include the potential for dispersal following gemmule ingestion (McAuley and Longcore, 1988). More recently these methods were expanded by Green and Figuerola (2005), suggesting more information is required to understand the relative importance bird-mediated dispersal in structuring invertebrate populations. Once a habitat is colonised, mechanisms of sponge dispersal over short

distances may include drifting of fragments and/or gemmules to new substrate. Other invertebrates may facilitate this, for example, certain caddisfly larvae have cases that are largely or entirely covered by living sponge (Fig 1b). Not only does this enable sponge colonies to be mobile, it enables the caddisfly larvae to be inconspicuous to fish predators (Resh, 1976). Sexually produced sponge larvae may influence sponge persistence at a site depending upon factors such number and quality of larvae, availablilty of settling sites for larvae, and biotic events occurring during and after settlement (Grosberg, 1981) (Chapter 5, Table 5). It is assumed that freshwater sponge larvae are neutrally buoyant and are dispersed using cilia and water currents (Bergquist, 1978). However, sponge larval dispersal has not been researched in the UK and was not possible during the present study due to the low number of larvae observations.

Several dispersal methods were suggested to account for the unusual amphiatlantic distribution of *R. ryderii* while addressing the fifth key question. Previously, the use of morphological taxonomic keys and/or molecular data to identify *R. ryderii* in UK habitats has been difficult due to the paucity of information. However, despite a thorough analysis of *R. ryderii* colonies using several sources of data (surveys in UK, samples from Ireland, and Museum samples), the number of *R. ryderii* colonies was low compared to *S. lacustris* colonies (in Chapter 5), but consistent with the relative species occurrence in the UK (Chapter 2). While ideally the present analysis would have involved a larger sample size, results were the first to indicate rotule size differences in *R. ryderii* gemmuloscleres. Colonies of *R. ryderii* were only associated with *S. lacustris* and were not located in canal habitats. This suggests that isolation of *R. ryderii* populations by ecology may be more important than isolation. Isolation and connectivity are part of freshwater spatial dynamics and the present study has demonstrated that this affects the number of species found. Thus the analysis of all sponge species, including rare species, is important to investigate the effect of habitat changes regardless a sponge species' dispersal ability. This work (Chapter 6) has clarified the taxonomy of *R. ryderii* so identifying this species should be easier and there is the potential for refining distribution data.

Much of the taxonomic confusion concerning *R. ryderii* involved megasclere variability. Although the present study did not investigate the presice mechanisms of morphological variation, future PCR cloning and sequencing investigations could target genes involved in spicule formation, for example genes responsible for the up-regulation of the enzyme silicatein (Krasko *et al*., 2000). When investigating the forth questions, spicule differences in *S. lacustris* (Chapter 5) reflected responses to different sub-site environments. Investigating how silicatein functions at different steps of spicule production and details of silicon metabolism in sponge cells may help to elucidate this response. Such investigations could also clarify the array of spicule character used in sponge taxonomy (Fromont and Bergquist, 1990). Further work is needed on the impact of environmental variables on *R. ryderii* acclimation and the usefulness of morphological traits for taxonomy.

Sequence data in the present work (Chapters 2, 4, 5, 6) was hampered because isolating uncontaminated genetic material is especially difficult in freshwater sponges. This is because a) life cycle changes mean that only a small amount of sponge material may be available; b) sponges are inhabited by a variety of epibionts and/or symbionts including Bryozoa, Insecta, Arachnida, Mollusca, Oligochaeta, Hirundinea, and Nematoda (Gugel, 2001; Matteson and Zacobi, 1980; Procter and Harvey, 1998; Traveset, 1990; Wilkinson, 1978) many of which are microscopic and/or camouflaged so are difficult to remove; and c) sponge feeding results in large numbers of bacterial cells inside sponge tissue. Extracted DNA often includes large amounts of non-target genetic material that was either co-amplified or preferentially amplified during PCR (Erpenbeck *et al*., 2002). Verification of each sequence was important for this reason. Extensive surveys also revealed the possibilities that different species of sponge can grow over each other, so methods included taking multiple samples from a colony to ensure it was one species.

Conclusion

Both genetic approaches and spicule morphology can provide sponge taxonomic information. Using morphological characteristics has been a traditional method to identify species (Packer *et al*., 2009). This approach started with Aristotle and became organized due to Linnaeus (Radulovici *et al*. 2010), but can have four

significant limitations (see Hebert *et al*., 2003a) especially for sponge species identification. First, both phenotypic variation in body shape (see Chapter 2), and environmentally induced spicule variation (see Chapter 5) can lead to incorrect identification. Assessing spicule variation has been critical in determining the distribution of the UK species *Racekiela ryderii* (see Chapter 6). Second, sponge morphology involves a limited numbers of characters and a degree of homoplasy (e.g. smooth oxea megascleres of *S. lacustris, E. fluviatilis* and *Eu. fragilis*, see Chapter 4) leave some species' delineation unresolved (see Hooper and van Soest, 2002). Thirdly, freshwater sponge classification relies heavily on gemmulosclere characteristics (Penney and Racek 1968; Reiswig *et al*., 2009). However gemmules are not present in all species (e.g. *Pachydictyum spp* see Chapter 4) or at all life history stages. Lastly, before the present work, there was a paucity of suitable taxonomic keys, and all keys require some level of expertise in sponge terminology (Will and Rubinoff, 2004). Accurately using taxonomic keys may require either (i) specific microscopic methods for observations of spicule morphology from adult sponge tissue or (ii) time consuming methods to transform adult sponge tissue to gemmules.

Genetic sequence data together with morphology can provide valuable insights and contribute to the formation of an integrated picture of biodiversity in natural and anthropogenic UK freshwater habitats. If taxonomy studies are carried out for biomonitoring and environmental change investigations then molecular methods of identification may offer optimal results. However, when investigating freshwater ecosystem functioning, then traditional taxonomic methods may be appropriate. This thesis included multidisciplinary approaches to address species diversity, species distribution, abundance, key water parameters, spicule variability, sequence divergence, and molecular and morphological taxonomy. These are areas of freshwater sponge ecology that have been relatively neglected in the literature. Although no single water parameter was important for all species it is now clear that the morphology and abundance of freshwater sponge species are linked to environmental conditions in UK inland waters. Thus sponges have the potential to be an "early warning system" for changes in ecosystem functioning. The resultant data

add fundamentally to the understanding of the genetics and ecology of freshwater sponges, and contribute to canal ecology.

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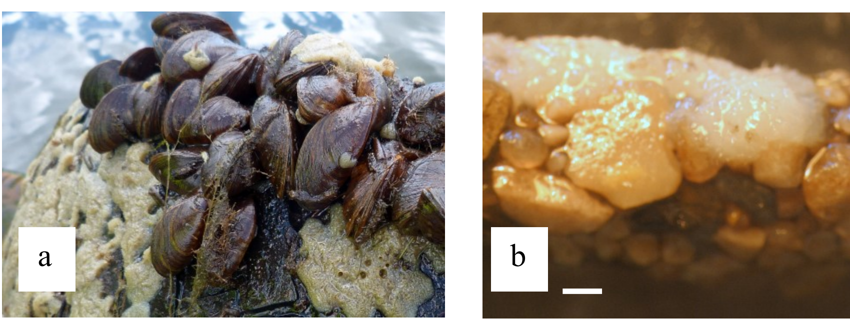

Fig. 1 a) An association between freshwater sponges and zebra mussels taken from a lake habitat as part of the UK survey (Chapter 2). b) Small *E. fluviatilis* colony (white) growing on caddisfly case (scale bar=1 mm).