UNIVERSITY OF SASSARI

Dissertation for the Degree of Doctor of Architecture and Environment
presented at Sassari University in 2019

XXXI Cycle

Ecological surveys for the valorization of spring environments of Sardinia: implications for their fruition and environmental protection.

PH.D. CANDIDATE: ***Giuseppina Grazia Lai***

DIRECTOR OF THE SCHOOL: ***Prof. Vincenzo Pascucci***

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Prof. Marco Cantonati
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My faith is in you, Father almighty,
Creator of heaven and earth,
of the visible and invisible universe

To my parents and to my dear friend Bruno

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Abstract

Springs are ecosystems with unique features and great ecological value. In the Mediterranean area they are more exposed to the risks of alteration of their structure and natural functioning because of direct and indirect human pressures and climate change. Despite this, springs remain poorly studied and specific approaches of management and protection are still missing. The main purpose of this PhD was to acquire knowledge on ecology of karst and thermo-mineral springs of Sardinia, traditionally neglected. The research activities focused on diatom microflora from different substrates in 17 springs of the Island. The results indicated a high diatom biodiversity, also highlighted by the presence of *Sellaphora gologonica sp. nov.* and *Chamaepinnularia thermophila*, the latter observed only in very few sites in the world so far. The analysis of the relationships between species and environmental variables, confirmed that diatoms can reflect the effects of important factors related to the vulnerability of these ecosystems, such as hydrological stability (flow permanence), discharge, climate change (extreme flash floods) and nutrient enrichment. This thesis offer food for thought on the importance of enhancing and preserving these environments so important from an ecological point of view and of so large interests for human uses. The information collected may represent a starting point for the development of strategies aimed at their proper management.

KEYWORDS: Mediterranean springs, karst springs, thermo-mineral springs, Bacillariophyceae, diatom biodiversity, species distribution, ecology, climate change.

Riassunto

Le sorgenti sono ecosistemi con caratteristiche uniche e grande valore ecologico. Le sorgenti dell'area Mediterranea sono maggiormente esposte a rischi di alterazione della loro struttura e del loro funzionamento naturale a causa di pressioni antropiche dirette e indirette e dei cambiamenti climatici. Nonostante questo, le sorgenti rimangono poco studiate e mancano ancora approcci specifici di gestione e tutela. Lo scopo principale di questo dottorato è stato quello di acquisire prime conoscenze sull'ecologia di sorgenti carsiche e termo-minerali della Sardegna. Le attività di ricerca hanno riguardato 17 sorgenti dell'Isola e lo studio è stato focalizzato sulla microflora delle diatomee di diversi substrati. I risultati ottenuti hanno indicato un'elevata biodiversità, evidenziata anche dalla presenza di *Sellaphora gologonica sp. nov.* e *Chamaepinnularia thermophila*, finora osservata solo in pochissimi altri siti del Pianeta. L'analisi delle relazioni tra specie e variabili ambientali ha confermato che le diatomee possono riflettere importanti fattori legati alla vulnerabilità di questi ecosistemi, come stabilità idrologica (permanenza del flusso), portata, cambiamenti climatici (eventi alluvionali estremi) e arricchimento di nutrienti. Questa tesi offre spunti di riflessione sull'importanza di valorizzare e preservare questi ambienti così importanti da un punto di vista ecologico e di vasto interesse per gli usi umani. Le informazioni raccolte possono rappresentare un punto di partenza per lo sviluppo di strategie finalizzate a una loro corretta gestione.

PAROLE CHIAVE: sorgenti Mediterranee, sorgenti carsiche, sorgenti termo-minerali, Bacillariophyceae, biodiversità delle diatomee, distribuzione delle specie, ecologia, cambiamenti climatici.

1. Introduction

1.1. Springs: definition and general characteristics

Springs are freshwater ecosystems formed by the natural emergence of groundwater (Bohle 1995, Reiss & Chifflard 2015). They occur where the water table intersects with the earth's surface or groundwater reach the surface through rock faults, fractures or depressions (Death et al. 2004, Scarsbrook et al. 2007). From the hydrogeological/bio- speleological point of view, springs are a main drainage point of a subterranean hydrographic system. Their physical and chemical characteristics (e.g., discharge, flow permanence, temperature and water chemistry) as well as the presence of some organisms linked to the groundwater, are relatively stable compared to other superficial water ecosystems (Gooch & Glazier 1991, van der Kamp 1995) and are mainly determined by the hydrogeological properties of the parent aquifer (van der Kamp 1995, Cantonati et al. 2006). For this reason, springs have been included among the "Groundwater Dependent Ecosystems" (GDEs) (Eamus & Froend 2006) in the category "obligately dependent" (Naiman et al. 2006, Kløve et al. 2011).

From the hydrobiological point of view, springs are mainly the origin of running surface waters and can be integrated into the upper part of a stream system (headwater). In the longitudinal zonation of running waters, they occupy a specific zone namely crenal divided into two subzones: springhead (eucrenal) and springbrook (hypocrenal), characterized by differences in structural and environmental parameters and in species composition (Reiss & Chifflard 2015). The boundaries of these subzones are not easily definable because the changes occur gradually and vary depending on the variables (Scarsbrook et al. 2007).

Springs are heterogeneous and very numerous environments on a global scale (Cantonati et al. 2012b, Glazier 2014). They have disjunct distributions and highly site-specific characteristics (Cantonati & Lange-Berlatot 2010, Cantonati et al. 2012b) and can be considered "island biotopes" o "water islands" (Werum 2001, Whittaker et al. 2001), capable of hosting specific and well differentiated biocoenoses (Cantonati et al. 2012b). From the ecological point of view, springs are 3-way ecotones that link ground and surface waters, spring and headwater stream, terrestrial and aquatic ecosystems in 4-dimensional framework: longitudinal, lateral, vertical, and temporal (Ward 1989, Scarsbrook et al. 2007, Cantonati et al. 2012b).

As ecotones, they have specific abiotic and biotic properties and processes (energy and material flow) and ensure interactions and exchanges important for the structural (e.g. community structure, biodiversity) and functional attributes (e.g. nutrient dynamics) of adjacent ecosystems (Naiman & Decamps 1997, Ward & Tockner 2001, Ward & Wiens 2001). Despite their usually limited dimension, springs are complex ecosystems characterized by different aquatic, semi-aquatic and semi-terrestrial microhabitats (e.g. mosses and debris layers) that build mosaic-like structures or patches (Cantonati et al. 2012b, Reiss & Chifflard 2015). This high structural complexity makes springs hotspots of aquatic biodiversity (Williams & Williams 1998, Ilmonen et al. 2012). Further, in pristine or relatively sheltered from heavy human impacts, springs can be an important source of high quality water and refuge for endemic, threatened and rare taxa because of their sensitivity to anthropogenic impacts (least-impaired habitat relicts) (Botosaneanu 1995, Cantonati et al. 2006, 2012b).

1.2. Main types of springs

Springs show a great variety of size and types and can be classified in numerous ways considering different aspects, including hydrologic regime, flow patterns, geologic setting, water temperature and chemistry (e.g., Desio 1973, Pentecost 2005, Cantonati et al. 2006, Scarsbrook et al. 2007, Kresic 2010, Glazier 2014).

Based on the hydrological regime they can be:

- *permanent*: flows present throughout the year, with any fluctuations of flow on a seasonal basis;
- *ebb-and-flow or periodic*: flows present in relatively uniform time intervals (periods);
- *ephemeral*: flows present only for a period of time (for example during major meteorological events), and absent at other times, reflecting directly the aquifer recharge pattern.

The classical classification based on eco-morphological characteristics such as water emergence type (punctual or areal) and current velocity conditions distinguishes the springs in (Steinmann 1915, Thienemann 1924):

- *limnocrene or pool springs*: water gathered in a pool; current velocity almost absent;
- *elocrene or seepage springs*: emergence of water from diffuse points forming a swampy zone; weak current velocity;
- *reocrene or flowing springs*: the water forms a streambrook, or a watercourse of I° order; rapid current velocity.

However, most springs belong to a large number of intermediate types (Schwoerbel 1959), that can be identified thanks to procedures considering mainly the substrate particle size (Cantonati et al. 2006).

Considering the geologic setting, the main types are:

− *volcanic springs*: these springs can be fed by different types of aquifers, in a variety of volcanic conditions (e.g., andesitic lava flows or fractured basalts). Springs usually originate where changes in the geologic substrate are present and their age is highly dependent on the history of the volcanic region. Volcanic springs generally have high permanence and hydrological stability (Scarsbrook et al. 2007);

− *alluvial springs*: these springs are fed by aquifers with a sedimentary origin (unconsolidated glacial and fluvial alluvium). The movement of water is facilitated by the porosity of the sediments. Alluvial springs often arise where groundwater are forced upwards by impermeable strata. The characteristics of the parent aquifer-springs draining shallow strongly control the permanence of alluvial springs and unconfined aquifers have, in general, lower permanence than artesian springs fed by deeper, confined aquifers (Scarsbrook et al. 2007);

− *karst springs*: these springs are fed by aquifers located on carbonate rocks, mainly limestones and dolomites. Because of their size and stability of the drained geological formations, karst springs tend to be permanent than other spring types (Scarsbrook et al. 2007). They are also characterized by a lower hydrological stability than other spring types. In fact, they reflect changes of flow in the catchment, as a result of high levels of connectivity between catchment, caves and conduits within the aquifer and the spring outlets (White 2005, Scarsbrook et al. 2007).

Based on the water temperature, springs can be distinguished in:

− *ambient springs*: the water temperature approximates the mean annual air temperature (MAAT) of a region;

− *cold springs*: the water temperature is below the MAAT;

− *thermal springs* (or *geothermal* or *superambient*) *springs*: water temperatures significantly higher than MAAT.

Thermal springs have been distinguished in several categories. However, it is commonly accepted that *hot springs* have temperatures exceeding human body temperature (~37–38 °C or 98–100 °F).

They are often associated with areas of active volcanism where the temperature of the spring water can be explained with the contact water/magma and chemical reactions that take place in depth (Waring 1965). Springs not associated with volcanic areas, mostly emerge in correspondence with crustal fault systems that allow the infiltration of meteoric water at depths of some kilometers from the surface, where they are heated, thanks to the geothermal gradient (Waring 1965). During its ascent, the water, with its heat content not yet dissipated, is enriched with a large quantity of mineral salts coming from the rocks. This process determines differences in the chemistry of water even within the same region (Waring 1965, Mwaura 1999).

Thermo-mineral springs are a special group of spring systems characterized by higher temperature values and electrolyte contents than other types of springs (Stavreva-Veselinovska & Todorovska 2010, Spahić & Temimović 2014). Overall, these springs are characterized by high concentrations of one or more ions (Cantonati et al. 2012b). They can have very different water chemistry, especially based on their prevalent ionic composition.

1.3. Importance of springs

Because of their abundance and widespread geographic distribution, springs are an important part of the hydrologic cycle and play a relevant role in the processes of erosion and transport of dissolved minerals (Glazier 2014). Springs are key habitats for biodiversity conservation (Cantonati et al. 2011, 2012b). As ecotones, they contribute to the maintenance of different surface aquatic ecosystems (Springer & Stevens 2009) and provide a wide range of ecological goods and services like moisture conditions, drinking water, food sources, minerals, shelter, thermal refuges, breeding sites and travel corridors for aquatic and terrestrial organisms (Glazier 2014). Springs are also important sources of high quality water. Karst springs, for example, provide good quality waters (Stevanović 2015) that may require light treatments to achieve compliance with drinking water standards (e.g., Koch et al. 2013).

According to UNESCO, they are the most significant and safest source of drinking water (Aureli 2010) and supply ~ 25% of the world's population (Ford & Williams 2007).

Thermo-mineral springs are exploited in industrial processing, heating settlements, agriculture and greenhouses, aquaculture, bottle water and the extraction of rare elements (Vimmerstedt 1998, Lund 2000, Lund & Freeston 2001, Baradács et al. 2001, Shevenell et al. 2002, Bahati 2003).

In several countries of the world, thermo-mineral waters are increasingly used in the health and well-being sector (Smith & Puczkò 2009) and in power generation (Lund et al. 2005, Petraccia et al. 2006), contributing to the control of greenhouse gas emissions of anthropogenic origin (Holm et al. 2012). Many springs are used for recreational purposes and are important tourist destinations (Glazier 2014). Finally, springs are important cultural resources and appear in the origination stories of many indigenous cultures (Stevens & Meretsky 2008). The establishment and survival of human populations in different arid regions of the world, from the Mojave Desert, USA (Sada & Sharpe 2004) to the Great Artesian Basin, Australia (Ponder 2004) and desert oases in Morocco (Ilahiane 1996) is strictly linked to springs. Historically, they have been revered as sacred and magical places and have been the focus of special religious celebrations and ceremonies and healing practices (Carmichael et al. 1994, Stevens & Meretsky 2008, Glazier 2014).

1.4. Springs as threatened habitats

Springs are among the most threatened ecosystems on Earth (Stevens & Meretsky 2008). They are particularly vulnerable to disturbance factors because of the small size and the presence of the fringing semiaquatic habitats (Cantonati et al. 2012b). Springs are affected by numerous direct and indirect anthropogenic activities, including land use practices in the catchment area (Table 1) (Danks & Williams 1991, Weigand 1998, Smith 2002). These impacts can cause a significant decrease of the natural springs, a deterioration of habitat and loss of characteristic communities (Danks & Williams 1991, Erman & Erman 1995, Howell et al. 1995, Cantonati et al. 2009). Water supply for drinking purposes, for example, has often involved the protection of the area around the spring (fencing), excavation of the spring mouth down to the bedrock (or aquifer) and construction of closed housing, including several deposition basins, mainly to avoid microbiological contamination of water (Cantonati et al. 2012b). The continuous water abstraction for drinking, irrigation and hydroelectric uses is the main threat for spring environments (Cantonati et al. 2012a). This threat will probably increase as results of variation in rainfall regime induced by climate change (Cantonati et al. 2007).

Table 1. Potential threats in spring ecosystems and possible impacts on water quantity and quality, physical habitat and biota (Source: Barquín & Scarsbrook 2008, modified). *CPOM = Coarse Particular Organic Matter.

Threats to spring habitats	**Potential impacts on spring habitats**
Groundwater abstraction	Excessive pumping lowers water table reducing spring flows
River regulation	Loss of interconnectivity with groundwaters may reduce spring flows
Afforestation	Increases sedimentation and evapotranspiration, and reduces groundwater recharge. Loss of riparian attributes reduces *CPOM, water quality and increases temperature, light and sediment load reaching the spring
Land drainage and urbanization	Groundwater recharge is diminished, lowering spring flows. Urban/road runoff also deteriorates spring water quality
Agriculture	Irrigation diminishes spring flows. The use of fertilizers and agrochemicals directly affects spring flora and fauna
Stock farming	Cattle erode banks and produce higher nutrient concentrations. It also deteriorates the substrate
Mining	Heavy metals and sediments directly affect spring flora and fauna. Water usage may also reduce spring flows
Landfill leachate and septic tanks	Chemical and faecal contamination of groundwater degrades spring water quality
Capture of springs	High transformation and simplification of the physical habitat reduces spring microhabitats
Recreational development and tourism	The physical habitat of the spring is transformed to provide swimming, fishing, and camping facilities. Heavy tramping may also damage the spring habitat
Introduction of exotic species	Elimination of native spring flora and fauna

Uncaptured springs are often compromised by nutrient enrichment, trampling cattle, sediment input, damage/removal of the surrounding vegetation and hydrological alterations due to inappropriate forest management (Weigand 1998, Cantonati et al. 2006, Fránková et al. 2009, Gesierich & Kofler 2010, Reiss 2011, Ilmonen et al. 2012). In many European regions, springs have disappeared and they are decreasing even in protected areas (Cantonati et al. 2012b). In the Mediterranean region, springs are particularly reactive to the hydrological cycle and affected by significant seasonal variations in the flow due to periods of intense precipitation and periods of prolonged drought (Meyer et al. 2003).

For this reason they are among the most vulnerable systems to extreme events and water over-exploitation potentially induced by climate change (Castellari et al. 2014). These aspects highlight the importance of a careful planning of their use, especially of water resource.

1.5. Legislative and management aspects

Springs are explicitly mentioned in environmental legislation only rarely in Europe.

In Finland, for example, springs have been identified as biodiversity hotspots in forested landscapes and are included among the thirteen woodland key habitats designated in the national Forest Act (Pykälä 2007). The European Union Directive of the 1992 (EU-HD 1992) on the conservation of natural habitats and of wild fauna and flora mentions only calcareous tufa springs as "Petrifying springs with tufa formation (Cratoneurion) 7220 priority habitat type" in Annex I: natural habitat types of community interest whose conservation requires the designation of special areas of conservation (Cantonati 2012b). In fact, nutrient enrichment and higher water temperature can prevent calcification and tufa formation in these springs and cause loss of characteristic taxa (e.g., Golubić et al. 2008, Sanders & Rott 2009, Rott et al. 2012). Springs are not covered by the European Union Water Framework Directive (EU-WFD 2000) and are scarcely considered in routine monitoring (Ilmonen et al. 2012). Their importance as sources of high quality water and biodiversity often seems to be underestimated, even in protected areas (Battegazzore 2012). The environmental protection of springs is still inadequate and further legislative attention and effort are needed to protect other types of springs (Battegazzore 2012, Cantonati et al 2012b).

Overall, management and conservation guidelines for springs are poorly developed or inexistent (Sada et al. 2005, Barquín & Scarsbrook 2008). Since the springs are environments at the interface between different ecosystems, protection and safeguard measures can be considered effective only if directed to maintaining the integrity of the habitat in all its components (Barquín & Scarsbrook 2008).

1.6. Springs of Sardinia

The first inventory of the springs of Sardinia was made between 1931 and 1932 to acquire first information on their number, location and some main characteristics (altitude, water temperature, discharge and uses) (Ministry of Public Works - Hydrographic Service 1934). This work, although not exhaustive, reported a total of 26,000 springs, including those with very low discharge, with 5,150 springs characterized by discharge ≥ 0.10 L sec^{-1} in the dry season. Among the latter, many springs located in an altitudinal range of 300-600 m a.s.l., were already used in those years for drinking water, irrigation and livestock breeding.

According to the regional technical map, springs currently inventoried in Sardinia are 10,109 (Fig. 1) (RAS, http://www.sardegnageoportale.it).

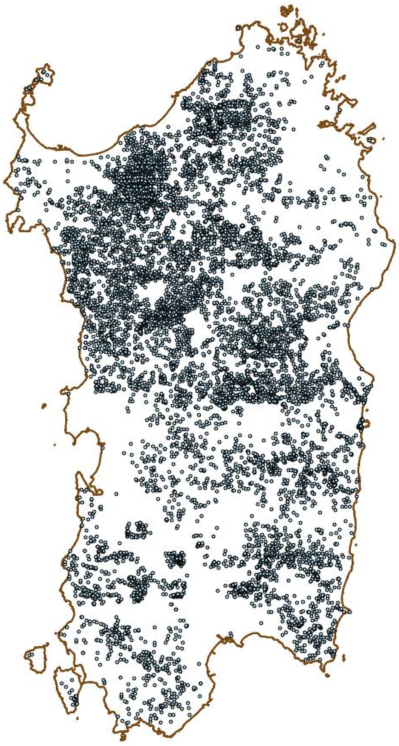

Fig. 1. Regional technical map of springs. Source: RAS, http://www.sardegnageoportale.it

Karst and thermo-mineral springs are two representative and important groups of springs of the Island.

Karst springs

Karst areas inventoried in Sardinia are 219 and cover a total area of 2,088 km^2 (8.7% of the total surface) from sea level up to 1,500 m in altitude (De Waele 2003, 2007). The most important aquifers are located below vast highlands named "Supramontes" in central-northern Sardinia, with some of the biggest springs of the Island (discharge >100 L sec^{-1}). Other important aquifers and springs are located in calcareous-dolomitic highlands named "Tacchi", in the central Sardinia, highland of Salto di Quirra and in the Iglesias region, in the southern part of the Island (De Waele & Murgia 2001).

Some springs with conspicuous discharge are also located in the Nurra and Logudoro regions, in the northern Sardinia. Many of these springs are captured for local drinking purposes. Despite the water supply of many areas of Sardinia depends almost exclusively on karst water, the current hydrogeological knowledge of karst springs are very fragmentary. For some areas, data on aquifers and springs are still completely missing (De Waele & Murgia 2001).Overall, the main impacts on the karst areas are due to mining, deforestation (especially in past centuries), fires, pastoral activities, urbanization, exploitation of aquifers, groundwater pollution, landfill, and tourism (hiking and caving activities) (De Waele 2007).

Thermo-mineral springs

Sardinia presents a relatively high heat flow density, probably due to the Tertiary geodynamics and radiogenic heating from the Variscan batholith. Recent data indicate that the high thermal flow from the basins can result from basin-wide heat redistribution by hot "granite" water flowing laterally in shallow aquifers (Cuccuru et al. 2015).

Thermo-mineral springs of Sardinia are generally located along a crustal faults system generated by the Cenozoic geodynamic (Cuccuru et al. 2015). The crustal faults are preferential routes for the rapid ascent of meteoric water, infiltrated at depths of some kilometers and heated by the relatively high thermal gradient (Fiorentino et al. 2017). The thermal areas are located almost exclusively in the western part of the Island, both in the northern and southern sectors (Dettori et al. 1982). In general, thermal landscape is characterized by two main kinds of waters: waters from granites (warmer) and waters from vulcanites (colder). According to available geochemical studies, thermal waters are characterized by a temperature range of 20-75 °C and different water chemistry, mainly bicarbonate-alkaline-earthy, chloride-sodium, chloride-bicarbonate-alkaline and chloride-alkaline-earthy (Dettori 1978a, b, Dettori et al. 1982).

A first inventory of springs of Sardinia (Ministry of Public Works - Hydrographic Service 1934) reported a total of 96 sites with water temperature >20 °C, mostly located at low altitude. Actually, only 52 springs are included in the inventory of national geothermal resources (Ministry of Economic Development - Department for Energy 2010).

Quantitative knowledge of thermal springs in Sardinia is incomplete and this resource is still largely unused (Brandis 1973, Cuccuru 2015).

According to the Regional Environmental Energy Plan (RAS 2013), the waters are mainly used for free thermal baths and curative and recreational purposes in seven spas. Some thermal springs such as the *Thermae Lesitanae* (Benetutti), *Thermae Neapolitanae* (Sardara) and *Aquae Ypsitanae* (Fordongianus) were known and frequented since Roman times and traces of this ancient history are still present in different areas (Puxeddu et al. 1934, Dettori et al. 1982, Fiorentino et al. 2017).

2. Diatoms

"Few objects are more beautiful than the minute siliceous cases of the diatomaceae: were these created that they might be examined and admired under the higher powers of the microscope?"
Charles Darwin ~ The Origin of Species, 1872

2.1. Diatoms: main morphological and biological characteristics

Diatoms (Division Heterocontophyta, Class Bacillariophyceae) are a group of eukaryotic and unicellular algae, sometimes colonial, with a size from 2 µm to about 500 µm (Mann 2017). These photosynthetic organisms possess chlorophylls *a* and *c* and accessory pigments such as fucoxanthin and β-carotene that give them a characteristic golden-brown color (Round 1990, Mann 2017).

A unique feature of diatoms is their siliceous cell wall or frustule (Fig. 2) that they build using dissolved silica from their natural environment. The frustule exhibits a wide variety of shapes and ornamentations and a glass-like consistency. For this reason diatoms are referred to as "algae in glass houses" (Pickett-Heaps & Pickett-Heaps 2003). The frustule is composed by two overlapping thecae (epitheca and hypotheca), consisting of large endpieces (valves) and a series of hoops (girdle bands) forming the cingulum. The girdle band nearest the valve is designated as valvocopula. Girdle bands are often split rings, sometimes with a tongue-like extension (ligula) that inserts between the ends of the adjacent band. The formation of the girdle bands during the life cycle allows the growth of the cell since the frustule is a rigid and not extensible structure (Pickett-Heaps et al. 1990, Round et al. 1990, Cox 2014). The valves, unlike the girdle bands generally smooth, are ornamented or perforated with striae composed by numerous small pores and arranged in species-specific patterns genetically determined (Round 1990, Saade & Bowler 2009). The frustule, transparent - opaline, allows the passage of light and in some cases has photonic crystals behavior arising from a regular and periodic distribution of pores (Cassaignon et al. 2014, Mann 2017). Diatoms use this structure to protect the cell by solar light and to optimize the photosynthetic process. Based on the shape of the frustule and patterns of the ornamentation, diatoms are traditionally distinct in Centric (valves predominantly circular and striae arranged in radiating rows) and Pennate (valves predominantly elongated and striae arranged in two rows either side of a longitudinal bar or rib (sternum).

Some important components of the frustule are:

− *raphe*: longitudinal fissure probably involved in the movement and adhesion to substrates. It is always absent in Centric diatoms and in some genera of Pennate diatoms;

− *pores*: structures involved in the exchange of nutrients between the environment and the cell and the extrusion of mucopolysaccarides (e.g., for making stalks and pads);

− *spines*: structures involved in the union of cells (colonies) or in floating of individual cells in the plankton. Spines can also increase the resistance to grazing and potentially increase the access to nutrients.

Diatoms have also a unique reproductive strategy. They have a diplontic life cycle that consists of two phases, vegetative and sexual (Round 1990, Mann 2017). The vegetative phase (*2n*) highly dominates over the sexual phase during the life cycle. It consists of repeated mitotic divisions, during which each daughter cell receives a parental valve and builds the new valve inside the initial parental valve. Because of the rigidity of the frustule, the new valves formed are slightly smaller than the parental valves and this process leads to a gradual decrease of the population mean size over time. The cell size is restored through formation of a special cell, the auxospore (*2n*), formed mainly through the fusion of gametes (*n*), in the sexual reproduction. The auxospore develops a new frustule inside the perizonium (pectic membrane). In general, the reproduction is anisogamic (gametes with different morphology) in Centric diatoms and isogamic (gametes with same morphology) in Pennate diatoms.

In presence of adverse environmental conditions, some diatoms can form silica cysts or statospores which germinate and return to their normal vegetative state when the conditions are again favorable (Round et al. 1990, McQuoid & Hobson 1996).

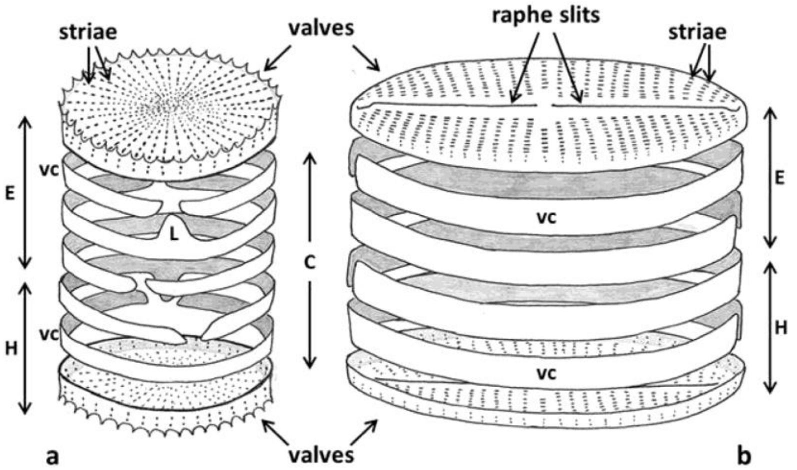

Fig. 2. Scheme of the basic structure of the frustule in a centric (a) and pennate (b) diatom. E = epitheca, H = hypotheca (H), C = cingulum. L = ligula, vc = valvocopula. Source: Cox 2014.

2.2. Habitats and ecology

Diatoms are a major group of organisms in terms of abundance, diversity and productivity of freshwater, brackish and marine ecosystems (Round 1990, Mann 2017). They play a significant role in silica and carbon biogeochemical cycles (Smol & Stoermer 2010) and are probably responsible for ca. 20–25% of the Earth's primary production (Mann 1999, Werner 1977). Diatoms are present with a large number of different species in almost all aquatic and most wet terrestrial habitats (Medlin 2016). According to Mann & Vanormelingen (2013) the total number of the extant species seems to be between 100,000 and 200,000. The biological success of diatoms can be attributed in part to the frustule (Pickett-Heaps & Pickett-Heaps 2003). In fact, it confers numerous advantages, among which a lower energetic cost compared to that required for the synthesis of an organic cell wall (Raven 1983) and defense against predators (Hamm et al. 2003). The species diversification across environments is a result of a combination of genetic, physiological and morphological factors (Falkowski et al. 2004). Diatoms exhibit an important diversity of life-forms and some taxa can present several successive life-forms during their life-cycle. According to Round (1990), Rimet & Bouchez (2012) and Mann (2017) life-forms can be distinguished, in general, in:

1. *Solitary cells*:

− Not attached: free-floating cells (e.g., Centric diatoms in plankton communities) or free-moving cells (e.g., *Nitzschia* or *Navicula* in benthic assemblages of the periphyton).

− Attached: benthic forms of the periphyton, mostly Pennate with different mechanisms of adhesion to different substrates:

- Adnate: cells attached by their valve face (e.g., *Cocconeis* species) or by their girdle (e.g., *Eunotia* species).
- Mucilage pad: cells stick to substrate with mucilage produced by a pole (e.g., *Diatoma* species).
- Mucilage stalk: cells produce a stalk through apical pore that sticks to the substrate. The stalk can be both linked to one cell (e.g., *Achnanthidium* species), or several cells (e.g., arbuscular colonies of *Gomphonema* species)

2. *Colonies*:

colonial forms are present both among planktonic and benthic species. In general, colonies can have many different forms (e.g., chain, ribbon, zig-zags, rosette, starlike, arbuscolar, mucous tubule) and the cells can be joined by different structures such as spines, mucilage excretions and stalks.

Attached diatoms, both single and colonies, live mainly on the following substrates:

- epipelon and endopelon: on surface and in the sediments;
- epipsammon: on the surface of the grains of sand;
- epilithon: on the hard and inert substrates (rock, stones, pebbles);
- epiphyton: on the surface of aquatic plants.

2.3. *Diatoms as indicators*

Diatoms possess biological and ecological attributes (e.g., ubiquitous presence in most habitats, sensitivity, motility, rapid life cycle, short resilience times) that make them excellent and reliable environmental indicators (e.g., Telford et al. 2002).

They respond to a number of environmental and biological variables (light, water temperature, substratum type, water velocity, mineral composition and content, nutrient availability, grazing) by shifting their assemblages composition and growth forms (Schönfelder 2000). According the causal principle (Schönfelder 2000) (Fig. 3), changes in diatom assemblages reflect the environmental changes in a more integrated way than traditional chemical methods.

Further, many taxa are cosmopolitan and have ecological optima and tolerances well documented, resulting useful indicators on wide geographic scale (Telford et al. 2002, Wojtal 2009). Diatoms can provide important information on long-term changes in aquatic environments starting from the Cretaceous (Telford et al. 2002). In fact, the composition of frustules preserved or fossilized in freshwater and marine sediments after the cell death, can reflect the species composition and environmental characteristics in the past (Battarbee 1986, Wojtal 2009). Variations in the composition of the community and indicator values may be summarized by means of functions, multivariate approaches and several indices and metrics (Mann 1999). The European Union Water Framework Directive (EU-WFD 2000) recognized the importance of these organisms including them among the biological indicators for the environmental assessment of freshwater ecosystems.

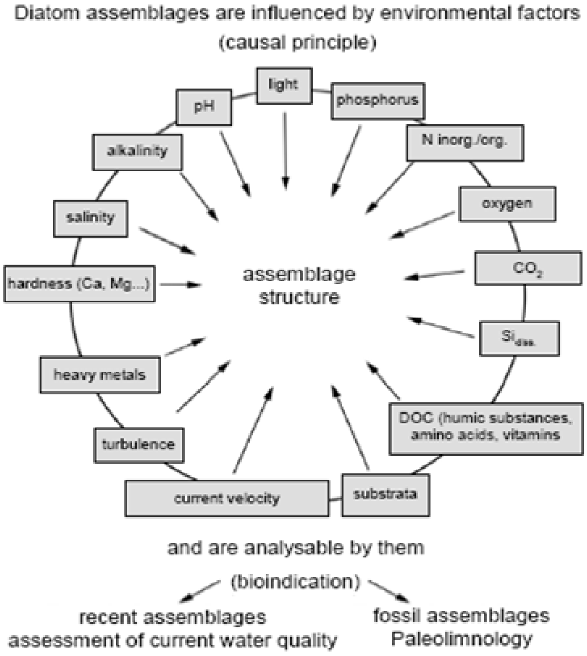

Fig. 3. The causal principle. Source: Schönfelder 2000.

2.4. Study of diatoms by microscopy

The use of diatoms as indicators of past and present changes in aquatic ecosystems requires an accurate identification of the species and an adequate understanding of their ecology and geographic distribution (e.g., Birks 1994, Mann 1999, Kociolek & Stoermer 2001).

The main obstacle in the use of diatoms is the difficulty of their taxonomic identification, often required at specific or subspecific levels (Blanco et al. 2017). Since the early times of investigation (early 19th century), the study of diatoms is mainly based on the morphologic analysis of the frustule by light microscope. In fact, while the protoplast is scarcely informative, the frustule possesses a number of diagnostic characters (e.g., symmetry and outline, length and width of the valves, presence/absence of raphe, disposition and density of striae) useful for the species identification. The study by light microscope (LM) involves limits in the identification of species due to the presence of small specimens with structures not clearly visible and species complexes that share similar diagnostic features (e.g., species in *Aulacoseira* or small fragilarioid species), extremely difficult to discriminate (Morales 2001).

Their differentiation often requires considerable expertise and in some cases can be very difficult, if not impossible (Morales 2001). Diatoms also present intrinsic morphological variability within species or even within infraspecific taxa (varieties, subspecies, forms). In fact, the cell size, shape of the frustule and number of striae of some taxa are subject to variation over the life cycle (Cox 2014). LM observations can be complicated by the fact that the already small frustule sizes are further reduced during asexual reproduction. As a result, many of the features of the cell wall approach or fall below the limit of resolution of the light microscope leading to confusion during their identification (Morales 2001). Further, the first formed (initial) valves can show atypical shape and striae arrangements because they reflect the shape of perizonium and full control of morphogenesis by cytoskeleton, which determines the positioning of some wall structures, is not re-established until the first vegetative division (Pickett-Heaps et al. 1990, Schmid 1994, Cox 2002, 2014). Often, problematic species show overlaps in the size range of the frustule and the number of striae in 10 µm with very similar species. Analysis of large number of individuals could reveal trends in size reduction and variation of key features in some populations. However, in some cases this approach is not resolutive. For example, in sympatric populations these trends can only be tracked own to a certain size, after which similarities are markedly accentuated (Morales 2001).

Some morphometric studies showed that shapes of apices and central areas in raphid diatoms are more informative than traditional size measures with respect to identity in morphologically similar taxa (Veselá et al. 2009). The use of common morphometric parameters (valve length, width and striae density) are not sufficient to achieve a correct identification to the species level and is discouraged also in large sample size (high number of individuals) according to Blanco et al. (2017). The scanning electron microscopy (SEM) improves significantly the accuracy of species identifications. In fact, several important diagnostic features not observable in LM, have been revealed by SEM (since 1930's) (Hasle & Fryxell 1970) allowing to clarify the identity of many species and discover new ones. SEM information (image files or photographs) can be reinterpreted years later and used for comparisons or as basis for further studies. The intermittent occurrence of heterovalvate diatom frustules (presence of two morphologically different valves in a single frustule) reveals that diatoms are able to modify the morphology of the frustule to a greater or lesser extent under different conditions (Cox 2014).

Future experimental studies can help the understanding possibilities of phenotypic responses to particular environmental factors and support the ecological interpretation of morphological assemblages (Cox 2014).

2.5. Diatoms in springs

Springs host stable populations of different organisms, including microalgae (Cantonati et al. 2007). Vascular plants and bryophytes are the main producers of biomass in springs (e.g., Hájková & Hájek 2003, Mogna et al. 2015). However, diatoms are an algal group often abundant (Poulíčková et al. 2004) and have a key role in the functioning of these aquatic systems (Cantonati et al. 2006). Springs are relatively stable freshwater environments (e.g., Odum 1971) and several studies reported only limited seasonal changes in composition of their diatom assemblages (Kadlubowska 1985, Cox 1990, Cantonati 1998a, Werum 2001). Some important factors that influence diatom communities are pH, geochemistry of the substratum, conductivity, current velocity, temperature and shading (Cantonati et al. 2006). The role of substratum type (lithic or plants) for the distribution patterns of diatoms is still uncertain and subject to debate (Cantonati et al. 2012b).

In general, diatoms communities of springs are characterized by species frequently also found in headwaters (Cantonati 2001), which are not crenobiontic (i.e., living only in springs) (Round 1957) or necessarily restricted to pristine running waters (Cantonati & Pipp 2000). Assemblages confined to spring environments seem to occur in slowly flowing springs (Sabater & Roca 1990, Cantonati 1998a, Cantonati et al. 2006). On the contrary, rheocrenic spring with fast current favors assemblages characterized by rheophilous species widely distributed (Cantonati et al. 2006). In these springs, crenophilous species or little known taxa are usually subdominant or rare (Reichardt 1994, Cantonati 1998a).

Further, springs on siliceous substratum seem characterized by many taxa with boreo-alpine distribution, while those on limestone have many cosmopolitan species (Sabater & Roca 1992, Cantonati 1998a). Diatom assemblages from thermo-mineral springs are often significantly influenced by temperature, pH, conductivity and specific ions (e.g. Owen et al. 2008, Quintela et al. 2013, Leira et al. 2017, Pumas et al. 2018). They can include brackish and marine taxa (e.g., Nikulina & Kociolek 2011). Some taxa abundant and well adapted to particular conditions (e.g., high water temperature and salinity), are considered representative of these peculiar environments (e.g. Mannino 2007, Quintela et al. 2013, López-Sandoval 2016).

2.6. Studies on diatom flora from springs of Europe

Crenobiology as a special field of Limnology specifically dedicated to study spring ecosystems has been proposed since the early 60s (Illies & Botosaneanu 1963, Cantonati et al. 2012b). However, springs have been understudied habitats for a long time and their diatom flora has received little attention until recently (Wojtal et al. 2009, Cantonati et al. 2012a, b).

In Europe, studies have been carried out in Poland (Wojtal et al. 2009, Wojtal & Sobczyk 2012), Belgium (Denys & Oosterlynck 2015), Spain (Aboal et al. 1998, Penalta-Rodríguez & López-Rodríguez 2007, Delgado et. al. 2013), Pyrenees (Sabater & Roca 1990, 1992), Swiss (Taxböck et al. 2017), Austria (Gesierich & Kofler 2010), Carpathians (Poulíková et al. 2004, Fránková et al. 2009), Slovenia (Menegalija & Kosi 2008, Zelnik et al. 2018), Bosnia and Herzegovina (Kapetanović & Hafner 2007, Hafner 2008, Dedic et al. 2015).

Diatom communities of thermo-mineral springs have been investigated in Iceland (Krasske 1938, Biebl & Kusel-Fetzmann 1966, Villeneuve & Pienitz 1998, Owen et al. 2008), Spitsbergen-Norway (Krasske 1938), Azores (Brock & Brock 1967), France (Beauger et al. 2016, 2017), Republic of Macedonia (Stavreva- Veselinovska & Todorovska 2010), Spain (Leira et al. 2017), Greece (Economou-Amilli & Anagnostidis 1981). Overall, the number of studies is limited and the networking among individual research groups is still insufficient (Cantonati et al. 2012b).

In Italy, studies have been carried out mainly in the Alpine region (Dell'Uomo 1975, Cantonati 1998a, b, Cantonati & Ortler 1998, Cantonati 1999, Cantonati & Pipp 2000, Cantonati 2001, 2003, Cantonati et al. 2006, Cantonati & Spitale 2009, Angeli et al. 2010, Falasco & Bona 2011, Filippi et al. 2011, Spitale & Cantonati 2011, Battegazzore 2012, Cantonati et al. 2012a, Falasco et al. 2012, Spitale et al. 2012a, b, Mogna et al. 2015) and in the Apennines (Dell'Uomo 1986, Dell'Uomo & Torrisi 2000, Torrisi & Dell'Uomo 2001). These studies have addressed aspects concerning the species composition and the relations between assemblages and environmental variables. Other studies have focused attention on applicability and the utility of diatomic indices for the assessment of environmental quality (Battegazzore et al. 2003, 2004, Torrisi & Dell'Uomo 2009, Battegazzore 2012, Battegazzore & Morisi 2012). Some studies have been carried out in thermo-mineral springs in Umbria (Dell'Uomo 1986) and Sicily (Mannino 2007).

In Sardinia diatom flora from springs has been explored only occasionally (Dell'Uomo 1990, Lai et al. (2016, 2018a, b, in press) and is still largely unknown. Some studies highlighted the importance of a greater knowledge and understanding of the biocoenoses and ecological dynamics of springs in Sardinia, also considering the significant potential vulnerability of these ecosystems (De Waele & Murgia 2001, De Waele 2003b). Diatoms can provide relevant information on the environmental integrity of springs (Mogna et al. 2015). Moreover, Sardinia is located in the middle of the Mediterranean Basin, one of the major hotspot of plant biodiversity (Myers et al. 2000, Zachos & Habel 2011) and the geographic and ecological isolation is recognized as an important prerequisite for the potential presence of endemic diatom species (Lange-Bertalot et al. 2003).

3. Objectives and contents

The information currently available on spring systems of Sardinia mainly concerns hydrogeological, physical and chemical aspects and are limited to some hydrographic basins. The knowledge of the ecological aspects, traditionally neglected and still largely unknown, is an important prerequisite for their proper use and management. The main objective of this PhD was to implement the existing knowledge on karst and thermo-mineral springs, using an integrated study approach based on morphological, physical, chemical and biological aspects. In particular, I focused on benthic diatom communities, considered very sensitive to the environmental conditions and capable of providing a rich system of information on the ecological characteristics and integrity of freshwater ecosystems.

I analyzed aspects concerning the species composition and structure of the assemblages, the distribution of the species in different substrates and spring systems and the relationships between diatom species and environmental variables.

Some insights concerned taxonomic and biogeographic aspects and the role of diatoms as indicators of water quality, physical disturbance and extreme weather events.

The specific objectives and the results obtained are described and reported in the following chapters:

Chapter I provides ecological information on eight karst springs of Sardinia, examining for the first time species composition and structure of epilithic and epiphytic diatom assemblages and the influence of environmental factors on the species assemblages. This study will be submitted for publication in *Plant Biosystems*.

Chapter II compares thermo mineral springs in Auvergne (France) and Sardinia. It describes the composition and structure of diatom assemblages in individual springs within each geographic region, the degree of similarity/dissimilarity among springs within each region and between the two geographic regions and the relationships between species and environmental variables. This study will be submitted for publication in *PeerJ*.

Chapter III provides additional information and data on the ecology of *Chamaepinnularia thermophila* (Manguin) C.E.Wetzel & Ector and a first framework on the global geographic distribution of this species. In the chapter, a population of *C. thermophila* from the thermo-mineral spring San Giovanni Su Anzu is compared with a population of *Navicula tongatensis* Hustedt from the original material, in order to propose the synonymy of the two species. This study was accepted for publication in *Oceanological and Hydrobiological Studies* on 26-10-2018.

Chapter IV highlights the presence of a still largely unknown biodiversity in the investigated springs. It describes *Sellaphora gologonica* G.G. Lai, Ector & C.E. Wetzel *sp. nov.* from the karst spring Su Gologone. It documents the morphology of the species with LM and SEM, provides a morphological comparison with the most similar *Sellaphora* taxa and information on its ecology. This study was published in *Phytotaxa*.

Chapter V reports the effects of the extreme flash flood caused by the extra-tropical cyclone Cleopatra in November 2013 on the karst spring Su Gologone. The chapter extends the information on diatom assemblages with new data on a complete seasonal cycle and describes the variations in their composition and structure over time and the relationships between diatom species and environmental variables. The results suggest the importance of the proper management and preservation of this biotope, both for the high biodiversity and potential vulnerability to climate change. This study was published in *Inland Waters*.

Chapter VI underlines the importance of sampling different substrates of springs in order to document the diatom microflora in a more exhaustive way. It analyses the differences among assemblages collected from rock, cobbles and fine sediments and the most significant variables for diatom assemblages in one thermo-mineral spring of Sardinia: the Casteldoria spring. The chapter documents some abundant, frequent and rare taxa by LM and SEM. This study is in press in *Botany Letters.*

Chapter VII describes the environmental quality of the Su Gologone spring, one of the most important and renowned karst springs of Sardinia, on the basis of physical, chemical and microbiological variables and diatom-based indices. The epilithic diatom assemblages of the spring were investigated on seasonal scale and provided new information on the species present compared to a previous study carried out in 1990. Some diatom species were documented by light microscopy (LM) and scanning electron microscopy (SEM). This study was published in *Acta Botanica Croatica*.

4. Material and methods

4.1. Spring selection

The springs of this study have been selected in two steps:

1. extensive literature search, including the consultation of the WebGIS of the Sardinia Region (RAS, http://www.sardegnageoportale.it), the Regional Speleological Cadastre (RAS, https://www.catastospeleologicoregionale.sardegna.it), the Inventory of national geothermal resources (Ministry of Economic Development - Department for Energy 2010) and Maps of the Military Geographic Institute (I.G.M.).

Overall, the available information is fragmentary and at the moment, there are no exhaustive documents showing the localization and main characteristics of the springs and updated thematic maps;

2. inspections in the field with the support of geologists of the University of Sassari, State Forestry Corps and local connoisseurs. During these numerous inspections the geographic position and altitude of the sites were recorded with a GPS Garmin eTrex Vista HCx and a direct evaluation of the most suitable sites for sampling activities was made.

This thesis is based on the work done at 17 springs (nine karst springs and eight thermo-mineral springs) (Fig. 4), including one karst spring previously sampled, whose study continued during this PhD. Photographs of the springs studied are shown in Figures 5 and Figure 6 and their main characteristics are reported in Chapters I and II.

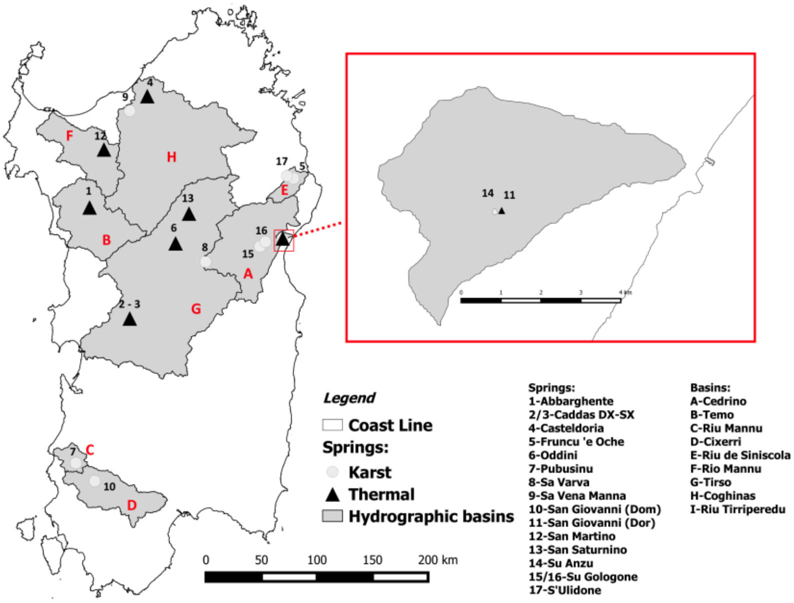

Fig. 4. Geographic position of the springs studied.

Fig. 5. Photographs of the karst springs studied and details of the water abstraction systems: S'Ulidone (a); Fruncu 'e Oche (b); Su Gologone - Sa Vena Manna (c-d); Su Gologone - Sa Vena (e); San Giovanni - Dorgali (f); Sa Varva (g); Sa Vena Manna (h); San Giovanni - Domusnovas (i); Pubusinu (j).

Fig. 6. Photographs of the thermo-mineral springs studied: San Giovanni Su Anzu (a); Caddas 1 (b); Caddas 2 (c); San Saturnino (d); Abbarghente (e); Su Banzu Mannu (f); San Martino (g); Casteldoria (h).

4.2. Measurements and sampling

Diatom and water samples were collected in summer 2016 and winter 2017 in 14 springs and on a whole annual cycle in two springs: Fruncu ‘e Oche (karst) and Casteldoria (thermo-mineral). Data from Sa Vena spring (Su Gologone karst system), processed in this thesis, derived from samplings carried out in winter 2010, spring 2011 and 2014, and winter and spring 2015. The diatom sampling was made on rock, cobbles, macrophytes and fine sediments, depending on the availability of substrates in each spring, by hard-bristled toothbrush and glass tubes in the entire spring area, following the methods reported in Kelly et al. (1998) and ISPRA (2014). Portions of floating algal mats were removed with sterile scalpels, following sampling methods reported in the literature (e.g., Ramsing et al. 2000, Ward et al. 2006). All samples were preserved in 100 ml polyethylene bottles and fixed *in situ* with a formaldehyde solution (4% v/v).

Water temperature in thermo-mineral springs was measured *in situ* with a WTW Multiline P4 and a digital thermometer (Temp 7 RTD basic with immersion probe PT100). In karst springs, water temperature, pH and conductivity were measured *in situ* with multiparameter probes (YSI ProPlus and YSI MPS 556).

Repeated measurements of discharge and water depth along transects, were made respectively by the volumetric method using a 1 l bottle and a chronometer and a graduated metric rod.

In all springs, water samples for physical and chemical analyses were collected using 1.5 l polyethylene bottles. The samples for dissolved-oxygen were collected in 150 ml glass bottles, and immediately fixed *in situ* with manganese sulfate and alkali-iodide-azide for the laboratory analyses (Winkler method). In karst springs, water samples for microbiological analyses (*Escherichia coli*) were collected in 150 ml sterile glass bottles.

4.3. Treatment and analysis of samples

Water samples

Physical, chemical and microbiological analyses were made at the laboratory following methods reported by APAT/IRSA–CNR (2003), APHA (1998), and Strickland & Parsons (1972). Some ions and trace elements of thermo-mineral waters, such as bicarbonate (HCO_3^-), carbonate (CO_3^{2-}), iodine (I^-), fluorine (F^-), bromine (Br^-), potassium (K^+), sodium (Na^+) and sulfate (SO_4^{2-}), were analysed in the laboratory of Ente Acque della Sardegna (EN.A.S) with methods of APAT/IRSA–CNR (2003).

Diatom samples

Diatom subsamples (50 ml) were treated after natural decantation for 48 h. The organic content of frustules was eliminated by an oxidation process on a heating plate with hydrogen peroxide H_2O_2 (30% v/v) and addition of HCl (37% v/v) to remove carbonates where necessary. After some washings with distilled water, a small amount of homogeneous sample (about 100 µl) was placed on microscope slides using Styrax resin (refractive index = 1.59).

Diatom observations and counts (~ 400 valves for each slide) were performed using a light microscope (LM, Zeiss Axiovert 10 and Leica DMRX equipped with phase–contrast and micrometric scale) at 1000× magnification. LM images were taken with a digital camera Axiocam Zeiss/ Leica DC500 mounted on microscopes.

For Scanning Electron Microscope (SEM) analysis, subsamples of the diatom suspension were air-dried on aluminium sheets and fixed on aluminium stubs that were sputter-coated with gold (Sputter Coater Edwards S-150A), examined using a Zeiss EVO LS10. A part of the subsamples, mounted on aluminium stubs and coated with platinum using a Modular High Vacuum Coating System BAL-TEC MED 020 (BAL-TEC AG, Balzers, Liechtenstein), was also analysed by an ultrahigh-resolution analytical field emission (FE) scanning electron microscope, Hitachi SU-70 (Hitachi High-Technologies Corporation, Japan).

Diatom species were mainly identified using Krammer & Lange-Bertalot (1986, 1988, 1991a, b), Krammer (2000), Krammer & Lange-Bertalot (2000), Lange-Bertalot et al. (2003), Werum & Lange-Bertalot (2004), Taylor et al. (2007), Levkov (2009), Żelazna-Wieczorek (2011), Wojtal (2013), Lange-Bertalot et al. (2017) and recent specialized literature.

4.4. Data processing and statistical analyses

The species observed in the samples were used to draw up floristic lists for each substrate and each spring. The ecological preferences of the species for pH, salinity, saprobity and trophic state were were attributed by referring to Dell'Uomo (2004), Torrisi & Dell'Uomo (2009) and Van Dam et al. (1994). First indications on the vulnerability status of the species were taken from the German Red List of threatened diatoms proposed by Lange-Bertalot & Steindorf (1996).

The structure of diatom assemblages was evaluated by species richness, Shannon-Wiener diversity index (H'; Shannon & Weaver 1949) and Pielou evenness index (J'; Pielou 1975) calculated by the OMNIDIA 6.0 software (Lecointe et al. 1993).

Diatom count data were converted into percentage relative abundances for statistical analyses.

Different tests and multivariate techniques were performed to explore variations of assemblages over time, similarity between assemblages, relationships between environmental variables and relationships between environmental variables and species using Primer 5 (Clarke & Gorley 2001), R 3.1.0 (R CORE TEAM 2012), R 3.1.3, R 3.2.2 and 3.4.1 (Venables et al. 2016) and Canoco 4.5 (ter Braak & Šmilauer 2002). Finally, the usefulness of diatom-based indices for the evaluation of the biological water quality (EPI-D) (Dell'Uomo 2004) and physical disturbance (NNS - NNS') (Battegazzore et al. 2003, 2004, 2007) was tested.

5. Chapter I

to be submitted to Plant Biosystems

Mediterranean karst springs: Diatom biodiversity hotspots under the pressure of climate change and nutrient enrichment

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Abstract

Karst springs are unique ecosystems increasingly threatened by anthropogenic pressures and climate change, especially in the Mediterranean area. In Sardinia (Western Mediterranean Sea, Italy), they are largely unexplored and their biocoenoses are mostly unknown. The diatom flora from two substrates (cobbles and macrophytes) in eight springs of different areas of the Island was investigated in summer 2016 and winter 2017. A total of 162 diatom taxa (58 genera) were found of which 27 (17 genera) only on cobbles and 26 (18 genera) only on macrophytes. The most abundant and frequent species were *Achnanthidium minutissimum*, *Amphora indistincta, Amphora pediculus, Cocconeis euglypta, C. pseudolineata, Kolbesia gessnerii, Planothidium frequentissimum*, and *P. lanceolatum.* Overall, 67 taxa (40 genera) were recorded in single sites and some of these taxa showed high affinity with specific environmental conditions. Hydrological stability (permanence of flow), discharge and nutrients ($P\text{-}PO_4^{3-}$ and $N\text{-}NO_3^-$) were the main environmental factors influencing diatom assemblages.

Our results suggest that diatoms can reflect important local factors related to the vulnerability of these spring ecosystems and underline the importance of their preservation both for biodiversity and water quality.

KEYWORDS: Bacillariophyta, carbonate substrate, hydrological regime, hydrochemistry, epilithon, epiphyton

Introduction

Karst springs are important aquatic ecosystems that provide good quality water and are characterized by high levels of biodiversity (Cantonati et al. 2015; Stevanović 2015; Wehr and Sheath 2015). The special intrinsic characteristics of karst aquifers make them very sensitive ecosystems to natural and anthropogenic pressures (Ravbar et al. 2015; Parise et al. 2018). The interest in the exploitation of karst waters continues to increase worldwide (Fiorillo & Stevanović 2015) and their protection is a prior environmental target for water sustainable management in Europe, where more than 30% of the water supply is guaranteed from these resources (Foster et al. 2013). Mediterranean springs can be more exposed to the risks of pollution, water withdrawal, and biodiversity loss because of the effects of climate change and increasing use of water for drinking and irrigation purposes (e.g., Bolle 2003; IPCC 2007). A proper protection and management of these ecosystems requires adequate knowledge of their hydrogeological and ecological characteristics. However, in many countries, springs are poorly studied and many aspects must still be investigated (Bakalowicz 2015). In Sardinia (Italy), the second larger island in the Mediterranean, hydrogeological studies are available only for some karst systems (De Waele & Murgia 2001). According to De Waele (2007), many karst areas of the Island have been modified by human activities with increasing impacts in the last century. Anyway, ecological surveys based on biological communities (diatoms and macroinvertebrates) are quite recent and focused so far only on the smaller spring of the Su Gologone karst system, one of the most important and renowned of the Island (Dell'Uomo 1990; Cuccui et al. 2011; Lai et al. 2016, 2018a, b).

The aims of this study were: 1) to describe species composition and structure of diatom assemblages from karst springs of different areas of Sardinia; 2) to test the relationships between species and environmental variables.

Study sites

The springs studied (Fig. 1, Table 1) are located in different karst areas of Sardinia, including the "Supramontes" which host the deeper and most conspicuous aquifers of the Island (De Waele & Murgia 2001). The springs cover an altitudinal range of 55-780 m a.s.l. and are mostly rheocrenic and perennial systems, with seasonal variations in discharge due to irregular rainfall and/or water abstraction. The springs SGOL and VARV are the only ones classified as limno-rheocrenic and seasonal, respectively. The springs SGOL and SGDN have the status of Natural Monument that defines the areas of great naturalistic value for conservation and protection in Sardinia (Regional Law 31/1998). Four other springs are located in Sites of Community Importance (SIC) for the Mediterranean biogeographic region: ULID and FOCH (ITB 021107 "Monte Albo") SGOL (ITB022212 "Supramonte di Oliena, Orgosolo e Urzulei - Su Sercone") and VARV (ITB021156 "Monte Gonare"). Most of the springs studied are captured for drinking purposes and the original morphology is strongly modified at SVMS, FOCH, and SGDR. The main disturbances are activities related to agriculture, animal breeding, and tourism. Others possible influences may be due to the presence of decommissioned-mine dumps for SGDN and PUBU and active limestone and dolostone quarries for ULID and FOCH (De Waele 2007). The SGOL spring also undergoes periodic floods due to the overflow of the Pedra 'e Othoni dam on the Cedrino River after intense rainfall (De Waele 2008).

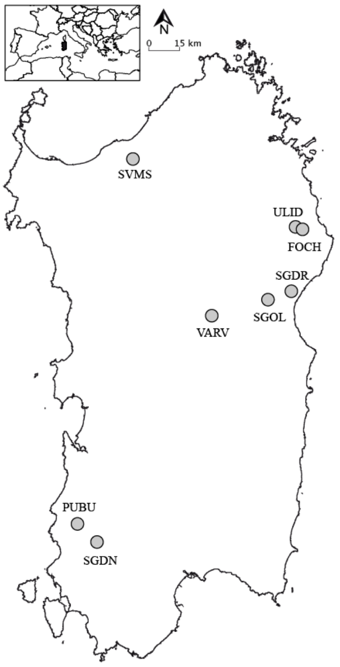

Fig. 1. Geographic position of the karst springs studied. Site codes: SVMS = Sa Vena Manna - Sedini; ULID = S'Ulidone; FOCH = Fruncu 'e Oche; SGDR = San Giovanni - Dorgali; SGOL = Su Gologone - Sa Vena Manna; VARV = Sa Varva; PUBU = Pubusinu; SGDN = San Giovanni - Domusnovas.

Table 1. Main characteristics of the springs studied. Type: R= rheocrenic; L-R = Limno-rheocrenic. Regime: P =Perennial; S= Seasonal.

Springs	Sigle	Altitude m a.s.l	Type	Regime	Water abstraction
S'Ulidone	ULID	606	R	P	X
Fruncu 'e Oche	FOCH	55	R	P	X
Su Gologone-Sa Vena Manna	SGOL	116	L-R	P	
San Giovanni	SGDR	168	R	P	X
Sa Varva	VARV	780	R	S	
Sa Vena Manna	SVMS	276	R	P	X
San Giovanni	SGDN	190	R	P	X
Pubusinu	PUBU	212	R	P	X

Methods

Sampling

Diatom and water samples were collected in summer 2016 and winter 2017. At VARV, the only sampling was carried out in winter because of the seasonal character of the spring (dry in summer). Epilithic diatoms were collected by scraping five cobbles with a hard-bristled toothbrush. Epiphytic diatoms were collected by squeezing submerged portions of different macrophytes, mainly the bryophyte *Brachythecium rivulare* Schimper, and the vascular plants *Nasturtium officinale* R. Br. subsp. *officinale* R. Brown and *Oenanthe* cf. *crocata* L. The material collected from the different macrophytes was integrated into a composite sample for each sampling and site. The sampling was made following the methods reported in Kelly et al. (1998) and ISPRA (2014). All samples were preserved in 100 mL polyethylene bottles and fixed *in situ* with a formaldehyde solution (4% v/v).

Water samples for physical and chemical analyses were collected using 1-L polyethylene bottles. The samples for dissolved-oxygen were collected in 150 mL glass bottles, and immediately fixed *in situ* for the laboratory analyses with the Winkler method (Winkler 1888).

Measurements and analyses

Geographic coordinates and altitude of the springs studied were recorded using a GPS Garmin eTrex Vista HCx. The water temperature, pH and conductivity were measured *in situ* with a multiparameter probe (YSI ProPlus). Hydrological Stability (HS), intended as permanence of flow on sampling dates, was recorded as 0) seasonal and 1) permanent. Repeated measurements of discharge (Q) were made downstream of abstraction systems and close to the diatom sampling points, by the volumetric method using a 1-L bottle and a chronometer. Physical and chemical analyses including oxygen, nutrients, and some major ions were analysed in laboratory using methods reported by APAT/IRSA & CNR (2003), APHA (1998), and Strickland and Parsons (1972).

Diatom subsamples were treated in the laboratory by an oxidation process on a heating plate with H_2O_2 (30% v/v) and addition of HCl (37% v/v) to remove carbonates. Cleaned diatoms were mounted on microscope slides using Styrax resin (refractive index = 1.59).

Diatom observations and counts (~ 400 valves for each slide) were performed using light microscopes (LM, Zeiss Axiovert 10 and Leica DMRX equipped with phase–contrast and micrometric scale) at 1000× magnification. Diatom species were identified consulting mainly Krammer & Lange-Bertalot (1986, 1988, 1991a, b, 2000), Lange-Bertalot et al. (2003, 2017), Werum & Lange-Bertalot (2004), Wojtal (2013) and Żelazna-Wieczorek (2011), and specialized literature (e.g., Cantonati & Lange-Bertalot 2009; Cejudo-Figueiras et al. 2011; Wetzel et al. 2015).

Data processing and statistical analyses

A Principal Component Analysis (PCA) was performed to identify patterns of variation in selected environmental variables and to express data similarities and differences among karst springs in summer and winter samplings. Data were log10 (x+1) transformed before analysis with Canoco 4.5 (ter Braak and Šmilauer 2002).

The structure of diatom assemblages was evaluated by species richness, Shannon-Wiener diversity index (H'; Shannon and Weaver 1949) and Pielou evenness index (J'; Pielou 1975) calculated by the OMNIDIA 6.0 software (Lecointe et al. 1993). Autecological preferences of diatom species for pH and moisture were attributed according to Van Dam et al. (1994). For salinity, saprobity and trophic state we referred to information for Italian running waters reported in Dell'Uomo (2004) and Torrisi & Dell'Uomo (2009). The taxa with a wider ecological range were equally placed between the respective autecological levels to obtain a simplified framework. The presence of vulnerable species was detected using the German Red List (Lange-Bertalot 1996).

Diatom count data were converted in relative abundances (RA) for the statistical analyses. Relationships between species and environmental variables were explored by the Redundance

Analysis (RDA) after a previous assessment of the length of gradient by a Detrended Correspondence Analysis of diatom data. The RDA was performed on the epilithic and epiphytic assemblages separately.

In both cases all species with relative abundance (RA) ≥1% were included in the analysis. Diatom and environmental data were $\log_{10}$ (x + 1) transformed. The significance of variables ($p < 0.05$) was assessed using Monte Carlo test simulations with 499 permutations. All the analyses were performed using Canoco 4.5 (ter Braak and Šmilauer 2002).

Results

Environmental variables

The mean values of physical and chemical variables for the springs studied are presented in Table 2. For the spring VARV the values shown (except for discharge) refer to the only sampling in winter, Overall, the sites showed a temperature range of 12.1–17.1 °C. The lowest values were measured at ULID, SGOL and VARV. The greatest seasonal variations of temperature were observed for SGDR (3 °C), SGDN (4 °C), and ULID (5.1 °C). The highest discharge values (> 0.6 L s^{-1}) were measured at FOCH and SGOL. pH values were slightly basic, except for a slightly acid value (6.43) recorded at ULID in summer. The water oxygenation was good with percentage of saturation ≥ 85% at all sites. Hardness and conductivity levels were intermediate-high with ranges of 148–350 mg $CaCO_3$ L^{-1} and 344–1150 µS cm^{-1}, respectively. Mg^{2+} was slightly more abundant than Ca^{2+} only at ULID and SGDN springs. Fe^{2+} and Mn^{2+} were present in very small amounts in all sites. N-NO_3^- was the most abundant inorganic nitrogen compound at all sites. The highest concentrations (>2000 µg N L^{-1}) were recorded at SGDR and SVMS, the same springs with total nitrogen >7000 µg N L^{-1}. SGDR and SVMS were also the springs with the highest concentrations of reactive phosphorus, respectively of 173 and 69 µg P L^{-1} and total phosphorus, respectively of 199 and 97 µg P L^{-1}. BOD_5 values were <1.5 mg O_2 L^{-1} at all sites. VARV and SGDN showed the highest values of total suspended solids.

The PCA analysis (Fig. 2) explained 69.3% of the total variance in the 2 first axes (53.1%: axis 1 and 16.1%: axis 2). The ordination of data distinguished the springs SGDR and SVMS from all the others on the basis of P-PO_4^{3-} and N-NO_3^-.

Table 2. Mean values of physical and chemical variables measured and analyzed in all springs studied. * = values of the only sampling in winter (except for discharge).

Variables/Springs	ULID	FOCH	SGOL	SGDR	*VARV	SVMS	SGDN	PUBU
T (°C)	12.1	15.6	13.1	15.1	13.2	17.1	16.2	16.1
Discharge (L s^{-1})	0.170	0.680	0.670	0.040	0.620	0.110	0.450	0.070
pH (units)	6.8	7.3	7.4	7.6	7.0	7.3	7.7	7.4
Alkalinity (meq L^{-1})	1.60	3.45	3.20	2.75	6.45	5.10	3.37	3.70
Cond (µS cm^{-1} at 25°C)	406	401	344	469	760	1150	519	613
DO (mg L^{-1})	10.6	10.9	10.6	9.5	9.0	9.0	10.1	9.3
DO (% saturation)	99	109	100	95	85	94	102	95
BOD_5 (mg O_2 L^{-1})	0.8	0.5	0.1	0.5	0.0	0.9	1.0	1.2
Cl^- (mg L^{-1})	46.1	30.1	21.3	40.8	42.5	195.0	60.3	56.7
Total hardness (mg $CaCO_3$ L^{-1})	148	178	157	158	350	348	198	220
Ca^{2+} (mg L^{-1})	21	34	32	40	56	55	28	42
Mg^{2+} (mg L^{-1})	23	22	20	20	51	51	31	28
Reactive phosphorus (µg P L^{-1})	10	12	9	173	8	69	8	7
Total phosphorus (µg P L^{-1})	22	38	21	199	48	97	60	28
$N\text{-}NO_2^-$ (µg N L^{-1})	2	1	3	2	1	6	1	1
$N\text{-}NO_3^-$ (µg N L^{-1})	26	502	764	2736	1549	3451	238	494
$N\text{-}NH_4^+$ (µg N L^{-1})	15	5	5	4	8	3	27	9
Total nitrogen (µg N L^{-1})	1318	1830	1237	7147	2216	7070	2494	1567
Reactive silica (mg L^{-1})	5.9	1.7	1.8	10.9	3.8	12.0	3.9	4.5
Fe^{2+} (mg L^{-1})	0.02	0.04	0.02	0.04	0.02	0.02	0.03	0.03
Mn^{2+} (mg L^{-1})	0.01	0.01	0.02	0.03	0.01	0.01	0.01	0.04
Total suspended solids (mg L^{-1})	0.5	0.5	2.0	3.5	10.5	1.7	15.7	1.4

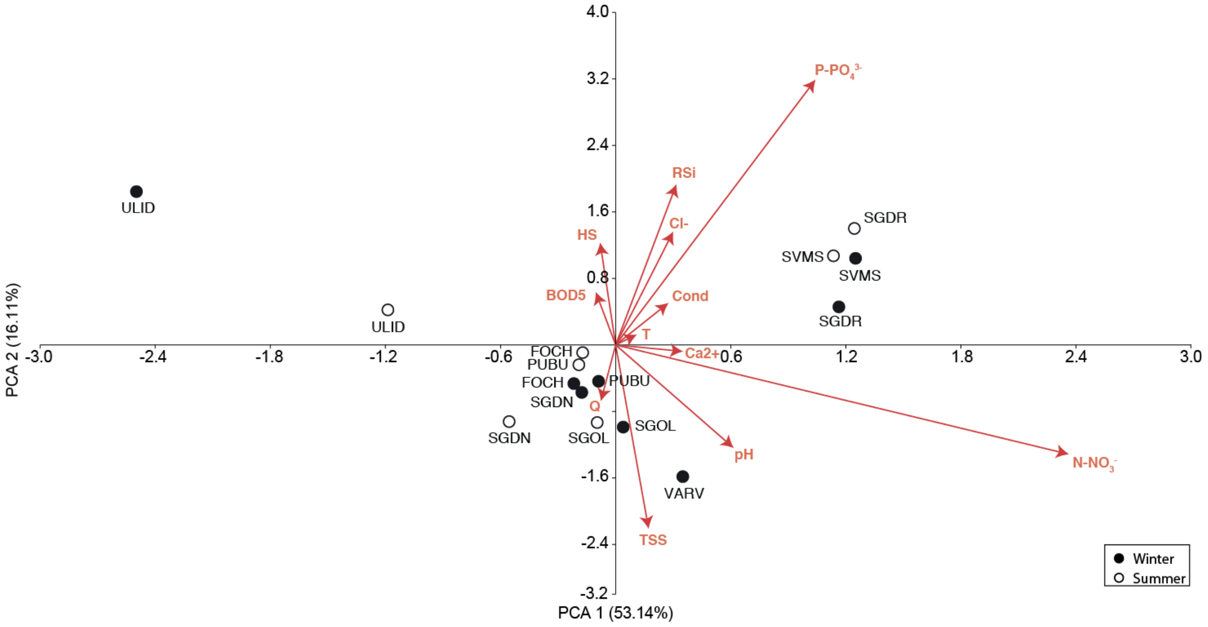

Fig. 2. Plot of the PCA analysis performed on selected environmental variables for all karst springs studied. Vectors = environmental variables; circles = spring sites. Site codes: SVMS = Sa Vena Manna - Sedini; ULID = S'Ulidone; FOCH = Fruncu 'e Oche; SGDR = San Giovanni - Dorgali; SGOL = Su Gologone - Sa Vena Manna; VARV = Sa Varva; PUBU = Pubusinu; SGDN = San Giovanni - Domusnovas.

Diatom species and ecological preferences

A total of 162 diatom taxa from 58 genera were found, of which 9 (7 genera) centric and 153 (51 genera) pennate. Overall, the most species-rich genera were *Navicula* (18), *Nitzschia* (14), and *Gomphonema* (11). The species observed only on one substrate were 27 taxa (17 genera) for cobbles and 26 (18 genera) for macrophytes. All abundant species (RA >5% in at least one sample) are listed in Table 3. Among these, the most frequent species (i.e., occurring in at least 4 springs) were *Achnanthidium minutissimum*, *Amphora indistincta*, *A. pediculus*, *Cocconeis euglypta*, *C. pseudolineata*, *Kolbesia gessneri*, *Planothidium frequentissimum*, and *P. lanceolatum*. Considering the whole data set, the taxa found in only one site were 67 from 40 genera (41% of the total species) (Table 4).

The most abundant were *Craticula buderi*, *Denticula subtilis*, *Gomphonema* aff. *cymbelliclinum*, *Humidophila perpusilla*, *Navicula veronensis*, and *Planothidium* sp.

According to the German Red List, 12 taxa (of a total of 103) can be considered vulnerable because included in threat categories: *Achnanthes inflata* and *Navicula vilaplanii* (Lange-Bertalot & Sabater) Lange-Bertalot & Sabater “rare”, *Amphora inariensis* and *Navicula striolata* “endangered”, and *Cocconeis neothumensis* Krammer, *Encyonema vulgare*, *Eunotia pectinalis* (Kützing) Rabenhorst, *Gyrosigma acuminatum*, *Halamphora normanii* (Rabenhorst) Levkov, *Grunowia solgensis*, and *G. tabellaria* “decreasing”.

The ecological information from the literature were available respectively for 99 taxa (pH) and 89 taxa (moisture, salinity, saprobity, and trophic state). The assemblages revealed the prevalence of alkalibiontic and alkaliphilous (71%), halophobous and oligohalobous (54%), xenosaprobic and oligosaprobic (51%) species. The trophic requirements highlighted a slight prevalence of species characteristic of eutrophic and hypereutrophic environments (38%) on the species characteristic of ultraoligotrophic and oligotrophic environments (35%). The share of typically mesotraphentic species was lower (24%).

With respect to preferences for moisture conditions, the majority of the species found (82%) are linked to the aquatic environment (categories 1-3). The species included in the category 4 “mainly occurring on wet and moist or temporarily dry places” were 14 (15% of the total). *Achnanthes coarctata* (Brébisson ex W. Smith) Grunow was the only taxon included in category 5 “nearly exclusively occurring outside water bodies”.

Table 3. List of the most abundant taxa (relative abundance >5% in at least one sample) found in the springs studied.

Diatom Taxa	ULID		FOCH		SGOL		SGDR		VARV		SVMS		SGDN		PUBU	
	C	M	C	M	C	M	C	M	C	M	C	M	C	M	C	M
Achnanthes inflata (Kützing) Grunow	0	0	0	3	0	0	1	1	0	0	0	0	0	0	0	0
Achnanthidium minutissimum (Kützing) Czarnecki	2	2	3	3	2	3	3	3	2	0	1	2	3	3	3	3
Achnanthidium subatomus (Hustedt) Lange-Bertalot	0	0	0	0	2	3	0	0	0	0	0	1	3	3	0	0
Amphora indistincta Levkov	3	3	3	3	3	3	2	1	0	0	2	2	1	0	0	0
Amphora pediculus (Kützing) Grunow	3	3	3	3	3	3	3	3	1	0	3	3	2	0	0	0
Caloneis fontinalis (Grunow) Cleve-Euler	1	1	1	2	3	2	1	1	0	0	2	3	0	0	0	0
Cocconeis euglypta Ehrenberg	1	2	3	3	3	3	3	3	0	0	3	3	3	3	3	3
Cocconeis pseudolineata (Geitler) Lange-Bertalot	3	0	1	0	3	0	0	0	0	0	1	1	3	0	2	0
Cosmioneis pusilla (W. Smith) D.G. Mann & A.J. Stickle	0	3	0	0	0	2	0	1	0	0	1	1	0	2	0	1
Craticula buderi (Hustedt) Lange-Bertalot	0	0	0	0	0	0	0	0	1	3	1	0	0	0	0	0
Denticula subtilis Grunow	0	0	0	0	0	0	0	0	0	0	3	0	0	0	0	0
Diploneis elliptica (Kützing) Cleve	1	1	1	1	1	2	0	1	0	0	1	1	1	0	3	1
Ellerbeckia arenaria (Moore ex Ralfs) R.M. Crawford	0	0	0	0	2	2	3	2	0	0	1	0	0	0	0	0
Encyonema vulgare Krammer	1	2	0	0	0	1	0	0	0	0	0	0	0	0	3	2
Eunotia minor (Kützing) Grunow	1	0	2	1	0	0	1	1	0	0	0	1	3	3	0	1
Fallacia sublucidula (Hustedt) D.G. Mann	0	0	0	0	3	1	0	0	0	0	1	1	0	0	0	0
Fragilaria candidagilae Almeida, C. Delgado, Novais & S. Blanco	2	3	0	0	0	0	0	0	0	0	1	0	0	0	0	0
Gomphonema aff. *cymbelliclinum*	0	0	0	0	0	0	0	0	3	3	0	0	0	0	0	0
Gomphonema micropus Kützing	2	1	3	0	0	0	0	0	1	1	0	1	0	0	0	0
Gomphonema rosenstockianum Lange-Bertalot & Reichardt	3	3	0	0	0	0	0	0	0	0	0	0	2	3	0	0
Grunowia solgensis (A. Cleve) Aboal	0	0	2	3	1	2	1	1	0	0	0	0	0	0	0	0
Halamphora sp.2	0	0	0	0	0	0	0	0	0	0	0	0	0	1	2	3
Humidophila contenta R.L. Lowe, Kociolek, J.R. Johansen, Van de Vijver, Lange-Bertalot	2	3	2	2	0	1	1	1	0	1	3	3	0	0	1	1
Humidophila perpusilla (Grunow) Lowe, Kociolek, J.R. Johansen et al.	2	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Kolbesia gessneri (Hustedt) Aboal	0	0	3	2	0	1	3	3	0	0	3	3	0	0	0	0
Meridion circulare (Greville) C. Agardh	3	3	3	2	0	2	1	1	3	3	0	1	1	1	0	0
Nitzschia fonticola (Grunow) Grunow	0	0	0	0	0	1	3	3	0	0	1	0	0	0	0	0
Nitzschia inconspicua Grunow	1	1	3	0	0	2	1	1	0	0	3	1	1	0	1	0
Nitzschia recta Hantzsch ex Rabenhorst	2	0	0	0	0	0	1	0	0	0	3	1	0	0	0	0
Odontium mesodon (Kützing) Kützing	1	2	0	0	0	3	0	0	0	0	0	0	0	0	0	0
Planothidium frequentissimum (Lange-Bertalot) Lange-Bertalot	3	3	2	2	1	1	2	1	0	0	2	3	3	2	3	3
Planothidium lanceolatum (Brébisson ex Kützing) Lange-Bertalot	3	3	3	3	3	3	2	2	0	0	1	3	0	1	0	0
Planothidium sp.	0	0	0	0	0	0	0	0	0	0	0	0	3	3	0	0
Pseudostaurosira alvareziae Cejudo-Figueiras, E.A. Morales & Ector	0	0	0	0	0	0	3	3	0	0	3	2	0	0	0	0
Reimeria sinuata (W. Gregory) Kociolek & Stoermer	1	0	0	0	1	1	0	0	0	0	1	0	3	1	0	0
Rhoicosphenia abbreviata (C. Agardh) Lange-Bertalot	2	1	0	0	2	1	0	0	0	0	3	2	0	0	1	0
Rhopalodia gibba (Ehrenberg) Otto Müller	1	3	0	1	0	0	0	0	0	0	0	0	0	1	2	1
Sellaphora arvensis (Hustedt) C.E.Wetzel & Ector	0	0	0	1	0	3	0	0	0	0	0	0	0	0	0	0
Sellaphora nigri (De Notaris) C.E.Wetzel & Ector	0	0	3	3	0	1	3	2	0	0	0	0	0	0	1	0
Staurosira venter (Ehrenberg) Cleve & J.D. Möller	0	0	0	0	1	0	2	3	0	0	0	0	0	0	0	0
Ulnaria ulna (Nitzsch) Compère	1	2	2	2	0	0	2	1	0	0	2	3	2	2	1	1

Note: Relative abundance of diatoms: 0 = absence; 1 = <1%; 2 = 1–5%; 3 = >5%. C = cobbles; M = macrophytes.

Table 4. List of all taxa found at only one site.

Taxa /springs	ULID	VARV	SGDR	SGOL	SVMS	SGDN	FOCH	PUBU
Lemnicola exigua Grunow	1							
Achnanthidium straubianum (Lange-Bertalot) Lange-Bertalot							2	
Amphora copulata (Kützing) Schoeman & R.E.M. Archibald					1			
Amphora inariensis Krammer				1				
Amphora vetula Levkov				1				
Aulacoseira ambigua (Grunow) Simonsen				1				
Aulacoseira sp.	1							
Bacillaria paxillifera (O.F.Müller) T. Marsson					1			
Brachysira sp.						1		
Caloneis sp.2				1				
Cocconeis pediculus Ehrenberg					1			
Cosmioneis pusilla (W. Smith) D.G. Mann & A.J. Stickle					1			
Craticula buderi (Hustedt) Lange-Bertalot		3						
Crenotia thermalis (Rabenhorst) Wojtal								2
Cyclotella meneghiniana Kützing					1			
Denticula subtilis Grunow					3			
Denticula tenuis Kützing				1				
Encyonema sp.					1			
Epithemia sorex Kützing	1							
Eunotia glacialifalsa Lange-Bertalot				2				
Fallacia insociabilis (Krasske) D.G. Mann					1			
Fallacia pygmaea (Kützing) Stickle & D.G. Mann					1			
Fragilaria rumpens (Kützing) G.W.F. Carlson						1		
Geissleria gereckei Cantonati & Lange-Bertalot				1				
Gomphonema acuminatum Ehrenberg				1				
Gomphonema aff. *cymbelliclinum*		3						
Gomphonema olivaceum (Hornemann) Brébisson					1			
Gomphonema truncatum Ehrenberg				1				
Gomphosphaenia sp.				1				
Grunowia tabellaria (Grunow) Rabenhorst				1				
Gyrosigma acuminatum (Kützing) Rabenhorst					1			
Humidophila perpusilla (Grunow) Lowe, Kociolek, J.R. Johansen et al.	3							
Iconella helvetica (Brun) Ruck & Nakov								1

Table 4. Continued

Taxa /springs	ULID	VARV	SGDR	SGOL	SVMS	SGDN	FOCH	PUBU
Lemnicola hungarica (Grunow) Round & Basson			2					
Luticola acidoclinata Lange-Bertalot								1
Luticola nivalis (Ehrenberg) D.G. Mann					1			
Luticola sp.				1				
Luticola sp.2							1	
Mayamaea sp.							1	
Navicula aff. *simulata*					1			
Navicula capitatoradiata H. Germain					1			
Navicula gregaria Donkin					1			
Navicula lanceolata Ehrenberg					1			
Navicula reichardtiana Lange-Bertalot							1	
Navicula striolata (Grunow) Lange-Bertalot			1					
Navicula tenelloides Hustedt			1					
Navicula veronensis Lange-Bertalot & Cantonati							3	
Nitzschia dissipata (Kützing) Rabenhorst					1			
Nitzschia microcephala Grunow					1			
Nitzschia sociabilis Hustedt				1				
Nitzschia sp.					1			
Nitzschia umbonata (Ehrenberg) Lange-Bertalot					1			
Nitzschia vitrea G. Norman	1							
Planothidium sp.						3		
Pleurosira laevis (Ehrenberg) Compère			2					
Pseudostaurosira subsalina (Hustedt) E.A. Morales					2			
Rhopalodia operculata (C. Agardh) Håkanasson					1			
Sellaphora crassulexigua (E. Reichardt) C.E.Wetzel & Ector				1				
Sellaphora seminulum (Grunow) D.G. Mann			2					
Simonsenia delognei (Grunow) Lange-Bertalot				1				
Stephanodiscus neoastraea Håkansson & Hickel	1							
Cyclostephanos invisitatus (M.H. Hohn & Hellermann) E.C.Theriot et al.							1	
Surirella angusta Kützing					1			
Surirella brebissonii var. *kuetzingii* Krammer & Lange-Bertalot					1			
Tryblionella apiculata W. Gregory					1			
Tryblionella hungarica (Grunow) Frenguelli					1			
Ulnaria acus (Kützing) Aboal						1		

Note: Relative abundance of diatoms: 0 = absence; 1: = <1%; 2 = 1–5%; 3 = >5%. Brown = taxa found only on cobbles; green = taxa found only on macrophytes; black = taxa found on both substrates.

Community-structure and diversity indices

The seasonal species richness for each substrate in all springs is presented in Figures 3 and 4. In summer, species richness varied from 14 (PUBU) to 39 (SVMS) for the epilithic assemblages and from 9 (PUBU) to 50 (SVMS) for the epiphytic assemblages. In winter, the values varied from 8 (VARV) to 54 (SVMS) for the epilithic assemblages and from 6 (VARV) to 45 (SGDR) for the epiphytic assemblages.

The seasonal values of diversity (H') and evenness (J') are reported in Table 5. In summer, the diversity values (H') ranged from 2.25 (PUBU) to 3.90 (ULID) for the epilithic assemblages and from 1.40 (PUBU) to 4.32 (ULID) for the epiphytic assemblages. In winter, the values varied from 1.44 (VARV) to 4.48 (SVMS) for the epilithic assemblages and from 1.21 (VARV) to 4.33 (SGDR) for the epiphytic assemblages.

In summer, the evenness (J') ranged from 0.57 (SVMS) and 0.80 (FOCH) for the epilithitic assemblages and from 0.44 (PUBU) to 0.82 (ULID) for the epiphytic assemblages. In winter, the values varied from 0.48 to 0.81 for the epilithic assemblages and from 0.47 (VARV) to 0.79 (SGDR) for the epiphytic assemblages.

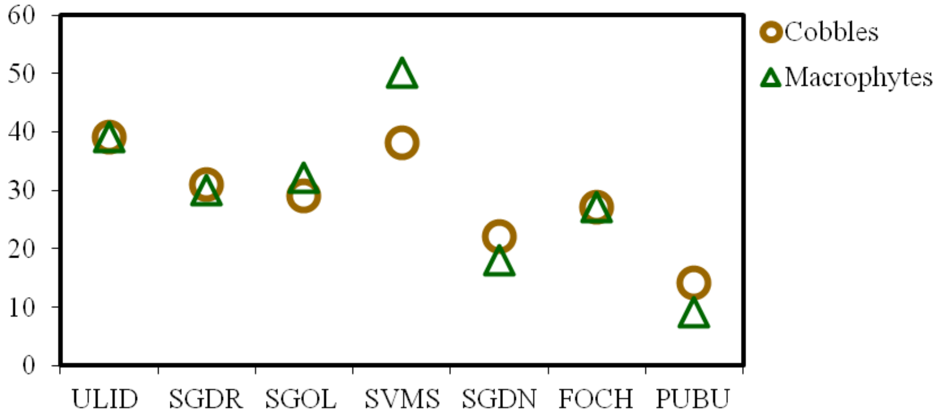

Fig. 3. Species richness of epilithic and epiphytic diatom assemblages in summer. Site codes: SVMS = Sa Vena Manna-Sedini; ULID = S'Ulidone; FOCH = Fruncu 'e Oche; SGDR = San Giovanni - Dorgali; SGOL = Su Gologone - Sa Vena Manna; PUBU = Pubusinu; SGDN = San Giovanni - Domusnovas.

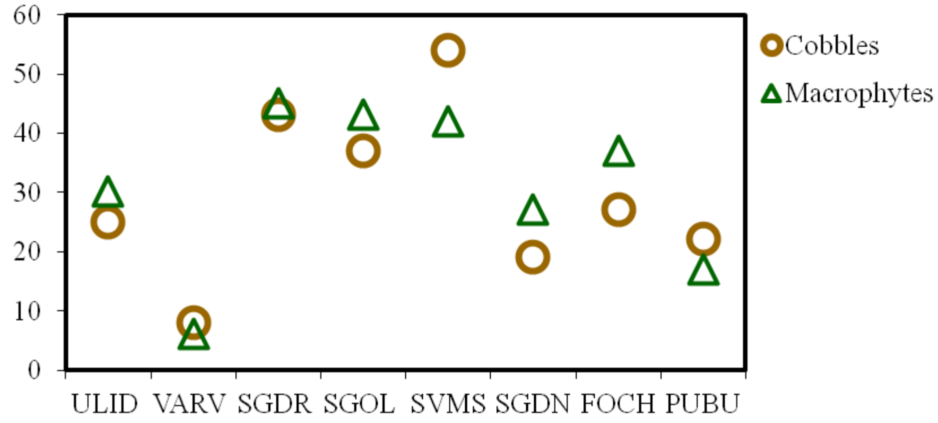

Fig. 4. Species richness of epilithic and epiphytic diatom assemblages in winter. Site codes: SVMS = Sa Vena Manna - Sedini; ULID = S'Ulidone; FOCH = Fruncu 'e Oche; SGDR = San Giovanni - Dorgali; SGOL = Su Gologone - Sa Vena Manna; VARV = Sa Varva; PUBU = Pubusinu; SGDN = San Giovanni - Domusnovas.

Table 5. Seasonal values of Shannon-Wiener diversity index (H') and and Pielou index (evenness) in all springs studied.

Sampling dates	Springs	H'	J'	H'	J'
		Cobbles		Macrophytes	
26/06/2017	ULID	3.90	0.74	4.32	0.82
18/07/2016	SGDR	3.69	0.74	3.77	0.77
18/07/2016	SGOL	3.22	0.66	3.32	0.66
20/09/2016	SVMS	2.98	0.57	4.03	0.71
22/09/2016	SGDN	3.25	0.73	2.82	0.68
18/07/2016	FOCH	3.80	0.80	3.60	0.76
07/07/2016	PUBU	2.25	0.59	1.40	0.44
27/02/2017	ULID	3.22	0.69	3.54	0.72
27/02/2017	VARV	1.44	0.48	1.21	0.47
15/12/2016	SGDR	4.37	0.81	4.33	0.79
15/12/2016	SGOL	4.05	0.78	3.95	0.73
30/01/2017	SVMS	4.48	0.78	3.89	0.72
31/01/2017	SGDN	2.21	0.53	2.90	0.62
24/01/2017	FOCH	3.77	0.79	3.89	0.75
31/01/2017	PUBU	2.93	0.66	2.28	0.56

Relationships with environmental variables

In the RDA analysis for the epilithic assemblages (Fig. 5), the first two axes accounted for 55% of the total variance of diatom species and environmental data (axis 1: 32.3% and axis 2: 22.7%). The significant variables for diatom assemblages were HS, $P\text{-}PO_4^{3-}$, and $N\text{-}NO_3^-$. *Caloneis fontinalis*, *Navicula tripunctata*, *Kolbesia gessnerii*, and *Pseudostaurosira alvareziae* from the SGDR and SVMS were associated with $P\text{-}PO_4^{3-}$ and $N\text{-}NO_3^-$ in axis 1. *Cocconeis euglypta* from SGOL spring was associated with HS in axis 2.

In the RDA analysis for the epiphytic assemblages (Fig. 6), the first two axes accounted for 54.9% of the total variance of diatom species and environmental data (axis 1: 28% and axis 2: 26.9%). $P\text{-}PO_4^{3-}$ and Q were the significant variables for the diatom assemblages. *Kolbesia gessnerii*, *Nitzschia fonticola* and *P. alvareziae* from SGDR and SVMS were associated with $P\text{-}PO_4^{3-}$ in axis 1. Axis 2 separated the samples collected from springs with highest Q values (FOCH and SGOL), with predominance of *Amphora indistincta* and *Planothidium lanceolatum* from springs with lowest Q values (ULID and VARV) with predominance of *Meridion circulare* and *Gomphonema* aff. *cymbelliclinum*.

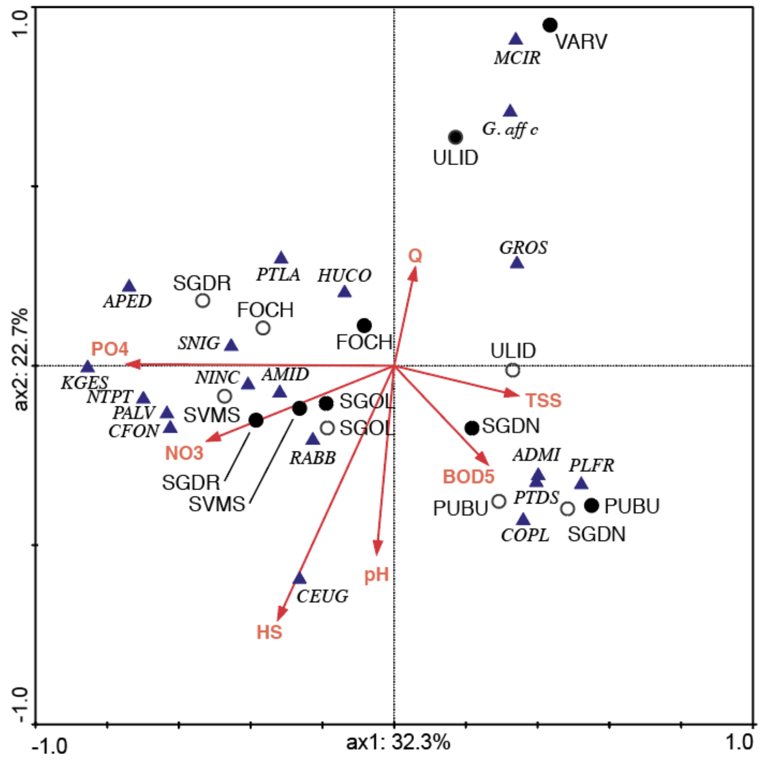

Fig. 5. RDA ordination plot for epilithic diatom assemblages. Vectors = environmental variables; circles = spring sites (black circles = winter samplings; white circles: summer samplings): triangles = diatom species. Species codes are reported in Table 2. ADMI = *Achnanthidium minutissimum*; AMID = *Amphora indistincta*; APED = *Amphora pediculus*; CFON = *Caloneis fontinalis*; CEUG = *Cocconeis euglypta*; COPL = *Cocconeis pseudolineata*; *G.* aff. *c.* = *Gomphonema* aff. *cymbelliclinum*; GROS = *Gomphonema rosenstockianum*; HUCO = *Humidophila contenta*; KGES = *Kolbesia gessnerii*; MCIR = *Meridion circulare*; NTPT = *Navicula tripunctata*; NINC = *Nitzschia inconspicua*; PLFR = *Planothidium frequentissimum*; PTLA = *Planothidium lanceolatum*; PTDS = *Planothidium* sp.; PALV = *Pseudostaurosira alvareziae*; RABB = *Rhoicosphaenia abbreviata*; SNIG = *Sellaphora nigri*. Site codes: SVMS = Sa Vena Manna - Sedini; ULID = S'Ulidone; FOCH = Fruncu 'e Oche; SGDR = San Giovanni - Dorgali; SGOL = Su Gologone - Sa Vena Manna; VARV = Sa Varva; PUBU = Pubusinu; SGDN = San Giovanni - Domusnovas.

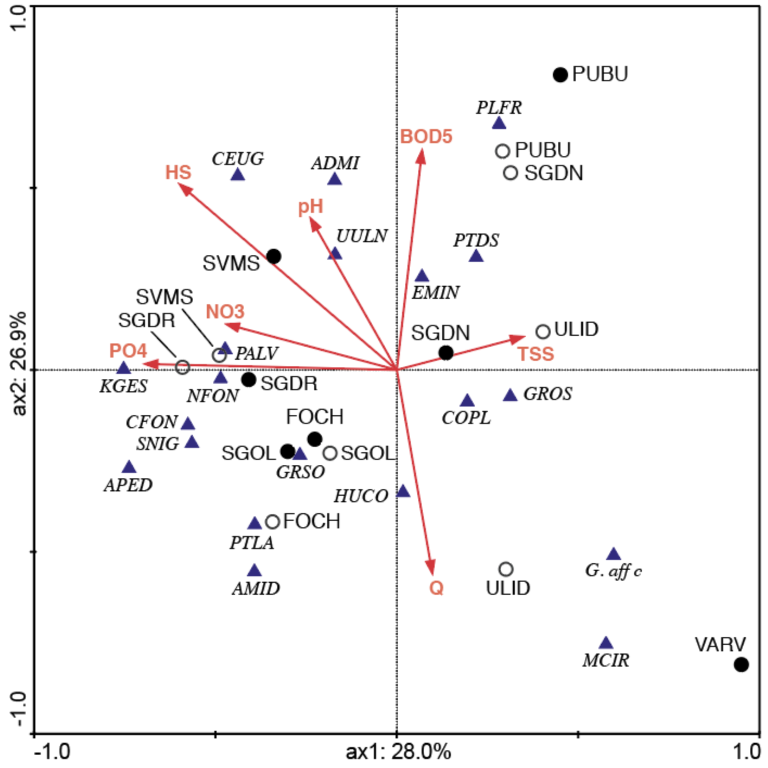

Fig. 6. RDA ordination plot for epiphytic diatom assemblages. Vectors = environmental variables; circles = spring sites (black circles = winter samplings; white circles: summer samplings); triangles = diatom species. Species codes are reported in Table 2. ADMI = *Achnanthidium minutissimum*; AMID = *Amphora indistincta*; APED = *Amphora pediculus*; CFON = *Caloneis fontinalis*; CEUG = *Cocconeis euglypta*; COPL = *Cocconeis pseudolineata*; EMIN = Eunotia minor; *G.* aff. *c.* = *Gomphonema* aff. *cymbelliclinum*; GROS = *Gomphonema rosenstockianum*; GRSO = Grunowia solgensis; HUCO = *Humidophila contenta*; KGES = *Kolbesia gessnerii*; MCIR = *Meridion circulare*; NFON = Nitzschia fonticola; PLFR = *Planothidium frequentissimum*; PTLA = *Planothidium lanceolatum*; PTDS = *Planothidium* sp.; PALV = *Pseudostaurosira alvareziae*; SNIG = *Sellaphora nigri*; UULN = *Ulnaria ulna*. Site codes: SVMS = Sa Vena Manna - Sedini; ULID = S'Ulidone; FOCH = Fruncu 'e Oche; SGDR = San Giovanni - Dorgali; SGOL = Su Gologone - Sa Vena Manna; VARV = Sa Varva; PUBU = Pubusinu; SGDN = San Giovanni - Domusnovas.

Discussion

Characteristics of the diatom assemblages

In the diatom flora of the springs studied, only few taxa are abundant and common to at least 4 sites: *Achnanthidium minutissimum*, *Amphora indistincta*, *Amphora pediculus*, *Cocconeis euglypta*, *C. pseudolineata*, *Kolbesia gessnerii*, *Planothidium frequentissimum*, and *P. lanceolatum.*

These taxa were also reported for the rheocrenic spring of the Su Gologone system in previous studies (Lai et al. 2016, 2018a). Most of these taxa were found in different springs and headwaters with calcareous substrate in the central Apennines (e.g., Torrisi & Dell'Uomo 2009). *Achnanthidium minutissimum* and *P. lanceolatum* were also reported as abundant and frequent members of diatom communities in Alpine springs (e.g., Cantonati 1998; Battegazzore et al. 2004; Gesierich and Kofler 2010; Falasco and Bona 2011). On the other hand, many taxa found in this study occurred at only one site suggesting a marked heterogeneity of assemblages. Similar findings were reported in studies on springs of the Alpine area in Italy and of the Beauce region in France (Bertrand et al. 1999; Cantonati & Spitale 2009). Some of these taxa showed high affinity with specific abiotic conditions observed in different sites based on the most recent information on the habitat and ecology of the species reported by Lange-Bertalot et al. (2017). For example, *Craticula. buderi* is consistent with the seasonal regime of the spring at VARV, *Crenotia thermalis* with the proximity of a decommissioned mine at PUBU, *Lemnicola hungarica* with the presence of *Callitriche stagnalis* Scop. (abundant in summer due to very low current velocity) at SGDR, and *Humidophila perpusilla* with shading at ULID. In addition, the presence of *Sellaphora seminulum* is consistent with the channeling at SGDR. Both epilithic and epiphytic assemblages included *Eunotia minor*, *Meridion circulare* and *Odontidium mesodon* considered as species with strong affinity to springs (crenophiles) (Gesierich and Kofler 2010; Mogna et al. 2015). In our study *O. mesodon* was observed less frequently than the other two taxa (only two sites). No significant differences in species composition were found between seasons suggesting a good stability of species assemblages. This is in agreement with limited seasonal changes in assemblage composition reported by several authors (eg., Kadlubowska 1985; Cox 1990; Cantonati 1998; Werum 2001).

Epilithic and epiphytic assemblages did not show significant differences even in species diversity but several species were observed only on one substrate similarly to other studies (e.g., Falasco & Bona 2011; Wojtal & Sobczyk 2012; Mogna et al. 2015). For example, *Amphora inariensis*, *Aulacoseira ambigua*, *Denticula subtilis*, *Halamphora montana* (Krasske) Levkov, *Geissleria gereckei*, *Iconella helvetica*, *Lemnicola exigua* Kulikovskiy, Witkowski & Pliński, *Navicula striolata*, *Nitzschia microcephala*, *Rhopalodia operculata*, *Sellaphora crassulexigua*, *Simonsenia delognei*, and *Surirella angusta* were found only in the epilithic assemblages. Other species like *Bacillaria paxillifera*, *Cyclostephanos dubius* (Hustedt) Round, *Epithemia sorex*, *Gomphonema acuminatum*, *Luticola nivalis*, *Navicula veronensis*, *Grunowia* *tabellaria*, *Sellaphora arvensis*, *Stephanodiscus neoastraea*, and *Ulnaria acus* were found only in the epiphytic assemblages.

Taxa not closely linked to the aquatic environment according to Van Dam et al. (1994) were found on both substrata. The occurrence of these taxa underlines the ecotonal nature of spring systems. *Achnanthes coarctata*, considered as pseudaerial species, was mostly found in epiphytic assemblages and seemed to be related to a greater abundance of mosses. According to several authors, carpets of mosses form ecotones between terrestrial and aquatic conditions increasing the heterogeneity of microhabitats (e.g., Cantonati 2004; Fránková et al. 2009; Bottazzi et al. 2011; Falasco & Bona 2011).

The vulnerable species according to the German Red List (rare, endangered, and decreasing) were mostly observed with low abundances and frequencies. The most abundant (RA >5% in at least one site) were *Achnanthes inflata*, *Encyonema vulgare*, and *Grunowia solgensis.* Based on the ecological information available, the vulnerable species found mostly prefer ultraoligotrophic environments, except for *Gyrosigma acuminatum*, characteristic of meso-eutrophic environments. However, the use of the Red List in Sardinia presents several limitations as already pointed out in a previous study (Lai et al. 2016). In fact, the Red List was compiled for a geographic area very different from the Mediterranean insular context. In addition the absence of historical data in the region prevents conclusions on the real level of vulnerability of the species. However, springs were recently confirmed by a study comparing large datasets of spring and stream diatoms to be refuges for sensitive and threatened diatom taxa (Taxböck et al. 2017), also likely to host Least-Impaired Habitat Relicts (LIHRe; Cantonati et al. 2012).

Biotic integrity

Overall, the studied springs showed high levels of biotic integrity (as suggested by species richness, diversity, and evenness). High species richness was found both in sites characterized by high naturalness, like ULID and SGOL, and strongly modified by water abstraction systems, like SGDR and SVMS. These results suggest that other factors besides the naturalness degree of the site, can contribute to the coexistence of numerous species. For example, the low current velocity and the greater availability of nutrients, might have favoured the high species richness at SGDR and SVMS. In fact, the fast current generally present in rheocrenic springs may favour rheophilic taxa, determining losses in species number and diversity (Sabater and Roca

1992; Cantonati 1998, 2001; Cantonati et al. 2012a). In addition, nutrient scarcity was indicated as the main reason for low species numbers in Alpine springs (Menegalija & Kosi 2008). By contrast, the low values of species richness at VARV spring, the only seasonal site investigated, suggest a role of the hydrological stability (permanance of flow) on diatom assemblages. Similar observations are reported for intermittent rivers and ephemeral streams where diatom communities show fewer species than perennial ecosystems (Tornés and Ruhí 2013). Periodic desiccation was also reported as a stressor affecting biodiversity in springs on carbonate substrata (Cantonati et al. 2012a). In our study, species richness was higher in the winter season in several springs. Similar results were reported for the epiphytic assemblages in the rheocrenic spring of the Su Gologone system (Lai et al. 2018) and other aquatic systems in the Mediterranean area (e.g., Solak et al. 2012). Winter is not the most favourable season for growth and diversity of diatom flora. However, as pointed out by Cantonati et al. (2012b), the biodiversity in springs may be a result of the presence of contrastingly disturbed microenvironments resulting from the mosaic nature of these ecosystems.

Relationships of diatoms with environmental variables

Overall, HS, Q, P-PO_4^{3-} and N-NO_3^- explained significant amounts of variance in diatom assemblages according to the RDA analyses. These results are supported by other studies in spring systems of different geographic areas, e.g.: karst springs of the southern limestone Alps in Slovenia (Menegalija & Kosi 2008), springs and streams of the south-eastern Alps in Italy (Cantonati & Spitale 2009), springs of the eastern Alps of Vorarlberg, in Austria (Gesierich and Kofler 2010) and karst springs in Poland (Wojtal and Sobczyk 2012). Moreover, hydrological factors, particularly flow permanence and discharge, and water chemistry are among the most important ecological factors determining species distribution and community according to Gerecke et al. (2011), and Cantonati et al. (2006, 2012b).

In our study, epilithic assemblages seem to be more sensitive to the flow permanence but better adapted to variations in discharge compared to epiphytic assemblages, probably as a result of their greater stability within the spring systems. In fact, unlike the cobbles, the macrophytes can remain more easily exposed or covered only by a thin film of water even for short times due to low discharge and water abstraction. Furthermore, epiphytic communities typically have a lower resistance to high discharge as compared to epilithic communities (Soininen and Eloranta, 2004). A more stable behaviour of epilithic assemblages than epiphytic assemblages over time was reported for the rheocrenic spring of the Su Gologone system in a previous study (Lai et al. 2018). Unlike the epilithic diatoms, only reactive phosphorus was significant for epiphytic assemblages. Among the macrophytes, carpets of mosses create conditions differing from those of the surface of stones for diatoms, including the availability of nutrients (Cantonati & Spitale 2009). However, the response to $P\text{-}PO_4^{3-}$ was identical for epilithic and epiphytic assemblages. Similar results are reported for a karst spring in Poland by Wojtal and Sobczyk (2012). These authors reported a very similar response of epilithic and bryophytic diatom assemblages to water chemistry, despite the preferences of some species for one substrate.

The results of RDA analyses for our springs are in good agreement with the ecological preferences of diatom species with respect to the trophic state of the water. The slight prevalence of species with preference for higher nutrient contents is consistent with values of $P\text{-}PO_4^{3-}$ and $N\text{-}NO_3^{-}$ that do not reflect the condition of pristine environments, especially in SGDR and SVMS. This aspect was also confirmed by the results of the PCA analysis that distinguished these two springs from all the others on the basis of nutrients.

Conclusions

This study provides a contribution to knowledge of the diatom flora from eight karst springs of Sardinia. In general, diatom assemblages showed high species richness both in sites characterized by high naturalness and in those strongly modified in their morphology by water abstraction systems.

The slight prevalence of species with preferences for higher nutrient contents is consistent with the nutrient inputs from pastures and agriculture present in the catchment areas. Significant relationships with hydrological stability (flow permanence), discharge, and nutrients ($P\text{-}PO_4^{3-}$ and $N\text{-}NO_3^{-}$), suggest that diatoms can reflect important factors related to the vulnerability of these ecosystems in the region. These results underline the need of a regular monitoring of their biodiversity and water quality and an adequate management aimed to preserve the good functioning of the ecosystem and to insure the desired ecosystem services.

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6. Chapter II

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Diversity, ecology and distribution of benthic diatoms in thermo-mineral springs of Auvergne (France) and Sardinia (Italy)

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Abstract

The diatom flora from thermo-mineral springs of Auvergne (France) and Sardinia (Italy) was investigated and compared. Samples were collected from rock/cobbles and fine sediments in 16 springs from January 2015 to March 2017. A total of 207 taxa (59 genera) were found. Multivariate analyses revealed significant differences in species composition and abundance among assemblages from each geographic region and assemblages from Auvergne and Sardinia (global R = 0.516; p = 0.2%) suggesting the importance of local and climatic factors in species distribution. Based on abundance and common occurrence in multiple sites, some taxa can be considered more representative for springs of each region: *Crenotia thermalis* for Auvergne and *Lemnicola exigua*, *Nitzschia amphibia*, *N. inconspicua* and *Rhopalodia operculata* for Sardinia. pH, conductivity, $N\text{-}NH_4^+$, $N\text{-}NO_3^-$ and HCO_3^- were the most significant environmental variables for diatom assemblages. Our results highlight the high heterogeneity of these spring systems and the presence of a partly still unknown biodiversity. Future taxonomic insights can be useful to define the identity of some abundant and sometimes dominant taxa not

identified to species level. Their identification is a crucial step for studies in similar ecosystems and for their ecological comparison.

Introduction

Thermo-mineral springs are a diverse group of aquatic environments and have characteristics that make them a very good prospect for different uses, including industrial processing, bottle water, power generation, health and well-being sector. The interest in this resource, used since ancient times, is greatly increased over the past few decades (Smith & Puczkò, 2009; Olivier, Venter & Jonker, 2011; Durowoju, Odiyo & Ekosse, 2017). The knowledge of the biotic communities of these springs, traditionally neglected compared to physical and chemical ones, is a prerequisite for their proper use and management (Boothroyd, Hay & Turner, 2006). In fact, the abundance and diversity of spring-dwelling organisms, besides having an intrinsic value, provide a number of ecological goods and services (Boothroyd, Hay & Turner, 2006; Mutia, 2010). Thermo-mineral springs are considered very interesting habitats for the study of algal microflora, including diatoms, which are often abundant and able to survive in different ecological niches (Leira, Meijide-Failde & Torres, 2017; Nikulina & Kociolek 2011). They can host distinctive assemblages of species, sometimes well adapted to extreme and hostile conditions (Quintela et al., 2013; Pumas, Pruetiworanan & Peerapornpisal, 2018). Further, diatoms are very sensitive to a variety of environmental conditions and can provide important information on the ecological integrity of these ecosystems (Owen, Renaut & Jones, 2008; Stavreva-Veselinovska & Todorovska, 2010; Niyatbekov & Barinova, 2018).

In Europe, investigations on diatom flora in thermo-mineral springs have been carried out in Czech Republic (Bílý, 1934; Lederer et al., 1998; Kaštovský & Komárek, 2001), France (Beauger et al., 2015; Beauger et al., 2016; Beauger et al., 2017), Iceland (Schwabe, 1933; Schwabe, 1936; Krasske, 1938; van der Werff, 1941; Biebl & Kusel-Fetzmann, 1966; Owen, Renaut & Jones, 2008), Italy (Dell'Uomo, 1986; Mannino, 2007; Lai et al. 2018), Republic of Macedonia (Stavreva-Veselinovska & Todorovska, 2010), Slovakia (Hindák & Hindáková, 2006; Hindák & Hindáková, 2007), and Spain (Leira, Meijide-Failde & Torres, 2017). Overall, these studies are still limited and focused on individual or multiple springs of specific geological settings while the comparison between different geographic areas is rare (e.g. Petersen, 1946; Owen, Renaut & Jones, 2008).

This study compares the diatom assemblages from thermo-mineral springs of Auvergne (Massif Central, France) and different areas of Sardinia (Italy). A crustal continuity between Sardinia and southwestern France (Provence) up to the Oligo-Miocene was hypothesized by several authors (e.g., Carmignani et al., 1994; Ziegler & Stampfli, 2001; Cassinis, Durand & Ronchi, 2003; Elter et al., 2004). In addition, paleontological affinities, particularly micro and macrofloras seem to confirm close relationships between Sardinia and southern border of France, including inner sectors of the Massif Central (Ronchi et al., 1998; Broutin et al., 1999; Broutin et al., 2000). Despite the possible common geological history, we hypothesized significant differences among diatom assemblages within each region and between Auvergne and Sardinia since both local and climate-related factors are considered important drivers for growth and distribution diatom species (e.g., Patrick, 1948). The main objectives of this study were: 1) to describe the species composition and structure of diatom assemblages; 2) to examine the degree of similarity/dissimilarity between springs in each geographic area and between Auvergne and Sardinia; (3) to explore the relationships between diatom taxa and environmental variables.

Materials & Methods

Study sites

The thermo-mineral springs studied in Auvergne (French Massif Central), are mainly located in the department of Puy-de-Dôme as it is the area with the majority of springs (Fig. 1, Table 1). The Massif Central is an old granitic shield formed during the Hercynian orogenesis. Then, with Alpine and Pyrenean orogenesis, dislocation of the shield appeared and volcanic formations occurred at the Tertiary and Quaternary periods (Mottet, 1999). The bedrock of the Massif is mainly granite and gneiss. At Châteauneuf-les-Bains and Les Martres-de-Veyre springs LEFO FDBL and PSAL come directly from granite. At Châtel-Guyon, Courpière and Augnat springs CHAT, BENE and CERI lie along a fault. At Boudes, spring BARD2 emerges from the fractured Hercynian metamorphic basement (Criaud & Fouillac, 1989; Négrel et al., 2000). The spring POIX1, at Clermont-Ferrand, emerges using a former volcano chimney crossing a layer of bitumen. Most of these springs have a high content of free CO_2 and CHAT, PSAL and POIX1 are sodium bicarbonate (Boineau & Maisonneuve, 1972). The majority of the selected springs, known since the Gallo-Roman period, are rheocrenic systems not used by local populations.

The thermo-mineral springs selected for Sardinia are located in the main geothermal areas of the central-northern part of the Island (Fig. 1; Table 1). It is not clear if these aquatic systems are linked to recent volcanic activity, but they are mostly located along a complex crustal faults system generated by the Cenozoic geodynamic (Minissale et al., 1999; Cuccuru, Oggiano & Funedda, 2015). This faults system form a plumbing network for water coming from deep-seated reservoirs heated due to the relatively high thermal gradient (Cuccuru, Oggiano & Funedda, 2015). At Fordongianus and Romana springs CAD1, CAD2 and ABB, are located on volcanic-sedimentary rocks. At Casteldoria and Benetutti springs CAS and SSA emerge from granitic-sedimentary rocks and at Oddini spring SBM from granitic rocks. At Codrongianos and at Dorgali springs SMA and SGA are respectively located on volcanic and carbonate substrates. These springs are mostly captured rheocrenic systems and have different water chemistry (e.g. Dettori, Zanzari & Zuddas, 1982). Gaseous emanations with prevalence of CO_2 are present at springs ABB and SMA and with prevalence of N_2 at CAS, CAD1, CAD2 and SBM (Dettori, Zanzari & Zuddas, 1982). The spring SMA is the only limnocrenic spring and differs from the others by the presence of significant incrustations of travertine and iron oxides (Dettori, Zanzari & Zuddas, 1982). Most of these springs, known since Roman times, are currently used as free thermal baths.

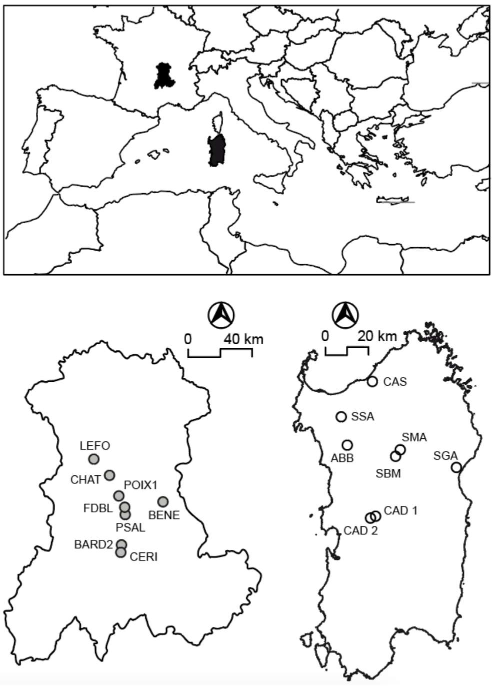

Fig. 1. Geographic position of the springs studied in Auvergne (France) and Sardinia (Italy).

Table 1. Main characteristics and use of the springs studied. Type: R = rheocrenic; R-M = rheocrenic modified; L = limnocrenic. Use: nu = not used; t = therapeutic; ftb = free thermal baths; d = drinking.

Spring	Abbreviation	Municipality	Altitude m a.s.l	Type	Captation	Use
Auvergne-France						
Petit Saladis	PSAL	Les Martres-de-Veyre	345	R		nu
Font de Bleix	FDBL	Les Martres-de-Veyre	332	R		nu
Bard 2	BARD2	Boudes	496	R		nu
Lefort	LEFO	Châteauneuf-les-Bains	379	R	X	nu
Cerisier	CERI	Augnat	497	R		nu
Châtel-Guyon	CHAT	Châtel-Guyon	421	R	X	t
Bénédictins	BENE	Courpière	331	R-M	X	nu
Poix 1	POIX1	Clermont-Ferrand	337	R		nu
Sardinia -Italy						
S. Giovanni Su Anzu	SGA	Dorgali	151	R	X	ftb
Caddas 1s	CAD1	Fordongianus	22	R		ftb
Caddas 1d	CAD2	Fordongianus	22	R		ftb
San Saturnino	SSA	Benetutti	252	R	X	ftb
Abbarghente	ABB	Romana	145	F	X	d
Su Banzu Mannu	SBM	Orani	205	R	X	ftb
San Martino	SMA	Codrongianos	211	L		nu
Casteldoria	CAS	Santa Maria Coghinas	7	R-M	X	t

Sampling

Diatom and water samples were collected from January 2015 to March 2017 from 8 springs of Auvergne and 8 springs of Sardinia.

The diatom sampling was made on rock/cobbles (depending on the availability of substrate) and fine sediments by hard-bristled toothbrush and glass tubes in the entire spring area, following the methods reported in Kelly et al. (1998) and ISPRA (2014). All samples were preserved in 100 mL polyethylene bottles and fixed in situ with a formaldehyde solution (4% v/v).

Water samples for physical and chemical analyses were collected using 1-L polyethylene bottles.

Measurements and analyses

The geographic position of the sites was taken using a DGPS Trimble Geo7x and a GPS Garmin eTrex Vista HCx. The water temperature was measured *in situ* with a WTW Multiline P4 and a digital thermometer (Temp 7 RTD basic with immersion probe PT100). Samples from Auvergne were analyzed by the high pressure ion chromatography technique. The samples were filtered using Whatmann GF/C filters. The ions were determined by Thermo Scientific Dionex ICS1100 and Thermo Scientific Dionex DX120 systems. Samples from Sardinia were analyzed following standard methods reported by APAT/IRSA & CNR (2003), APHA (1998) and Strickland & Parsons (1972).

Diatom subsamples were treated by an oxidation process on a heating plate with H_2O_2 (30% v/v) and if necessary HCl (37% v/v) was added to remove carbonates. Cleaned diatoms were mounted on microscope slides using Styrax (refractive index = 1.59) and Naphrax (refractive index = 1.73) resins. Diatom observations and counts were performed using light microscopy (LM, Zeiss Axiovert 10 and Leica DM2700M microscope equipped with phase-contrast and micrometric scale) at 1000× magnification. Subsamples mounted on aluminium stubs and coated with platinum were analyzed by a scanning electron microscope (SEM, Hitachi SU-70). Diatom species were identified using Krammer & Lange-Bertalot (1986), Krammer & Lange-Bertalot (1988), Krammer & Lange-Bertalot (1991a), Krammer & Lange-Bertalot (1991b), Krammer (2000), Krammer & Lange-Bertalot (2000), Lange-Bertalot et al. (2003), Werum & Lange-Bertalot (2004), Levkov (2009), Żelazna-Wieczorek (2011), and recent literature (e.g., Beauger et al., 2015; Wetzel et al., 2015; Beauger et al., 2016; Beauger et al., 2018).

Data processing and statistical analyses

The structure of diatom assemblages has been examined by species richness, Shannon-Wiener diversity index (H'; Shannon & Weaver, 1949) and Pielou evenness index (J'; Pielou, 1975), calculated by OMNIDIA 6.0 software (Lecointe, Coste & Prygiel, 1993). The ecological preferences of taxa respect to pH, salinity, trophic state and moisture were attributed consulting Van Dam, Mertens & Sinkeldam (1994).

Diatom count data were converted in relative abundances (RA) for the statistical analyses.

The similarity among assemblages was analysed by a non-metric multidimensional scaling ordination (nMDS).

A Bray-Curtis similarity matrix was constructed using log (x + 1) abundance data from all species with a relative abundance >1%. The significance of the differences was validated by a one-way analysis of similarities (ANOSIM). For this analysis, probability percentages (p) <0.03 were considered significant. To support ANOSIM, the percentage level of similarity of diatom assemblages and the percentage contribution of each species to the differences were determined by the SIMPER analysis. The species were classified from the highest to the lowest contribution to identify the sub-group whose cumulative percentage contribution reached 70% of the dissimilarity value. The nMDS, SIMPER and ANOSIM were performed by PRIMER 5 (Clarke & Gorley, 2001). A principal Component Analysis (PCA) was performed on environmental data to explore the explicative variables of the variance using Canoco 4.5 (ter Braak & Šmilauer, 2002).

The relationships between diatom species and environmental variables were explored by a Canonical Correspondence Analysis (CCA) using Canoco 4.5 (ter Braak & Šmilauer, 2002), after the previous assessment of the length of gradient (>4) by mean of a Detrended Correspondence Analysis of diatom data.

All canonical axes were used to assess the significant variables through analyses by means of a Monte Carlo test (1000 permutations). The data used to construct the environmental matrix were log(x+1) transformed. Ordination plots were generated both for sites and diatom species.

Results

Environmental variables

Results of physical and chemical variables for all springs are presented in Table 2. The sites showed heterogeneity in temperature, pH and conductivity values, with wider ranges for Sardinia than Auvergne. Springs with temperature ≤20 °C were FDBL, BARD2, CERI, BENE, POIX1 in Auvergne and SMA in Sardinia. pH ≥7.5 were measured at POIX1 in Auvergne and CAD1, CAD2, SBM and SSA in Sardinia. Conductivity values ≤ 1500 µS cm^{-1} were measured at FDBL in Auvergne and CAD1, CAD2, SBM, SGA, SSA in Sardinia. In Auvergne HCO_3^- was the most abundant at PSAL, FDBL, BARD2, LEFO, CERI, BENE, followed by Na^+ and Cl^- at PSAL, BARD2, CERI, BENE, Na^+ and SO_4^{2-} at LEFO and Ca^{2+} and SO_4^{2-} at FDBL. HCO_3^-, Na^+ and Cl^- were the main ions also at CHAT and POIX1, but Cl^- was the most abundant.

In Sardinia, Cl^- was the most abundant ion at CAS, CAD1, CAD2, SBM and SSA, whereas HCO_3^- was the most abundant at ABB, SGA and SMA. Other most abundant ions were Na^+ except for SGA, SO_4^{2-} at CAD1, CAD2, SBM and SSA, Mg^{2+} at CAS and SMA and Ca^{2+} at SGA. Overall, N-NO_3^- values were higher in springs of Auvergne (130-32029 µg L^{-1}) than Sardinia (<D.L.-2448 µg L^{-1}).

The PCA analysis (Fig. 2) performed on environmental variables explained 88.3% of the variance in the data in the 2 first axes (71.1%: axis 1 and 17.2%: axis 2). The ordination of data distinguished three main groups of springs: 1) most of the springs of Auvergne and ABB and SMA of Sardinia associated with higher values of Mg^{2+}, $HCO_3^-$and K^+, 2) POIX1 of Auvergne and CAS of Sardinia associated with Na^+ and Cl^- and 3) and CAD1, CAD2, SSA and SBM of Sardinia associated with pH.

Table 2. Results of physical and chemical variables measured and analyze in all studied springs.

	Auvergne-France								**Sardinia-Italy**							
Variable/Spring	PSAL	FDBL	BARD2	LEFO	CERI	CHAT	BENE	POIX1	ABB	CAST	CAD1	CAD2	SBM	SGA	SMA	SSA
T (°C)	20.7	18.0	14.2	32.6	18.1	27.3	14.7	13.3	22.1	71.5	49.3	53.0	32.2	29.3	11.2	20.9
pH	6.89	6.42	6.53	7.02	6.58	7.15	7.06	8.25	6.32	7.06	7.91	7.98	9.33	7.30	6.50	8.99
Conductivity (µS cm^{-1} at 25°C)	8540	1344	6510	4230	4040	8440	4120	4810	5270	8890	1152	1193	1020	590	4890	930
Na^+ (mg L^{-1})	1628	23	930	920	610	917	689	763	1539	1208	220	231	178	26	804	170
K^+ (mg L^{-1})	179.9	6.3	203.8	56.3	126.3	110.3	127.9	72.9	36.2	42.1	5.3	1.6	2.1	1.7	62.3	2.3
Mg^{2+} (mg L^{-1})	132.0	49.1	139.9	36.7	104.4	426.3	152.2	75.0	83.0	334.1	5.0	2.0	<D.L.	30.4	340.8	<D.L.
Ca^{2+} (mg L^{-1})	327	237	5	91	178	757	158	164	16	56	8	6	12	44	80	12
HCO_3^- (mg L^{-1})	2840	650	2013	1850	1870	2200	1910	610	3100	30.5	29.5	29.3	20.7	274.5	3044	17.1
F^- (mg L^{-1})	1.1	0.4	0.8	6.3	1.6	0.9	0.4	0.2	0.7	1.8	6.6	7.2	4.0	0.1	<D.L.	7.8
Cl^- (mg L^{-1})	1752	44	784	296	445	2419	293	1160	432	2794	284	305	269	46	262	220
Br^- (mg L^{-1})	5.1	0.0	4.0	1.4	0.2	5.3	0.1	2.0	0.9	8.2	0.7	0.7	0.7	0.4	<D.L.	0.5
SO_4^{2-} (mg L^{-1})	113	69	43	332	30	403	228	553	405	89	42	43	34	10	219	39
N-NH_4^+ (µg N L^{-1})	2.38	0.32	1.20	1.11	1.14	0.77	0.85	0.33	<D.L.	27	47	49	50	8	<D.L.	2
N-NO_2^- (µg N L^{-1})	<D.L.	<D.L.	0.02	0.01	0.01	0.02	0.02	0.01	1	1	1	1	<D.L.	6	6	1
N-NO_3^- (µg N L^{-1})	255	32029	386	662	506	130	704	210	7	81	567	14	<D.L.	2448	110	9

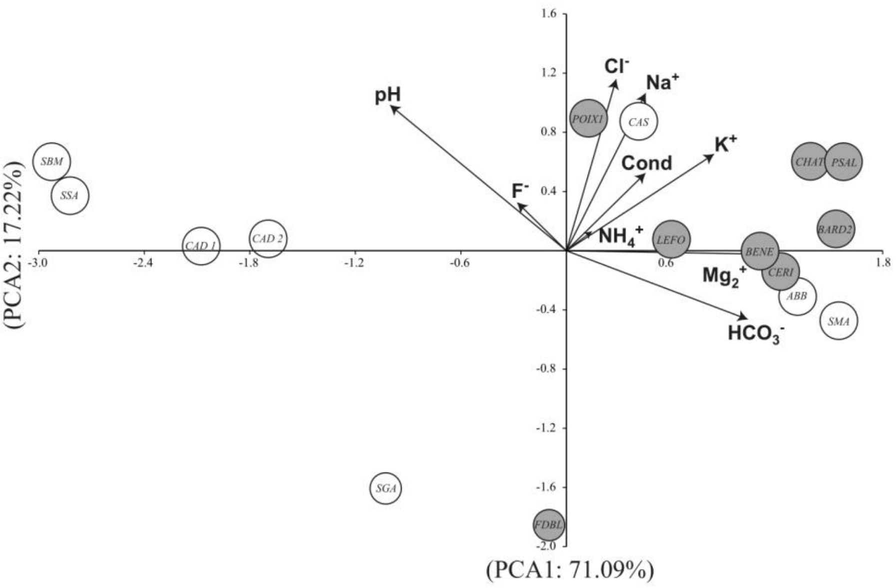

Fig. 2. Plot of the PCA analysis performed on environmental variables for all thermo-mineral springs studied. Gray circles = sites of Auvergne; white circles = sites of Sardinia. All sigles of sites are reported in Table 1.

Diatom assemblages

In total, 207 diatom taxa from 59 genera were found, of which 61 from 23 genera in the springs of Auvergne and 178 from 55 genera in the springs of Sardinia.

The species common to springs of Auvergne and Sardinia were 24 from 13 genera (11.6% of the total species) and included several *Navicula* (e.g., *N. cincta* (Ehrenberg) Ralfs, *N. veneta* Kützing, *N. sanctamargaritae* Beauger, *N. vilaplanii* (Lange-Bertalot & Sabater) Lange-Bertalot & Sabater) and *Nitzschia* species (e.g., *N.* aff. *liebethruthii*, *N. linearis* W. Smith, *N. inconspicua* Grunow and *N. microcephala* Grunow). Other common species were *Crenotia thermalis* (Rabenhorst) Wojtal, *Denticula subtilis* Grunow, *Diploneis elliptica* (Kützing) Cleve, *Halamphora normanii* (Rabenhorst) Levkov, *Gomphonema parvulum* Kützing, *Tryblionella debilis* Arnott ex O'Meara and *T. hungarica* (Grunow) Frenguelli.

Assemblages from Auvergne were composed by 12 taxa abundant (RA ≥5%), 8 taxa frequent (RA >1-5%) and 48 taxa rare (RA ≤1%).

The most species-rich genera were *Crenotia*, *Nitzschia*, *Navicula*, *Planothidium* and *Sellaphora*.

The most common species were *C. thermalis* (all sites except POIX1), *Planothidium frequentissimum* (Lange-Bertalot) Lange-Bertalot (6 of the 8 springs), *N. sanctamargaritae* and *N. veneta* (4 of the 8 springs).

Assemblages from Sardinia were composed by 5 taxa abundant (RA ≥5%), 11 taxa frequent (RA >1-5%) and 163 taxa rare (RA ≤1%). The most species-rich genera were *Nitzschia*, *Navicula* and *Gomphonema* with 27, 19 and 10 taxa respectively. The most common species were *N. veneta* (all sites), *Nitzschia amphibia* Grunow (7 of the 8 springs), *Lemnicola exigua* (Grunow) Kulikovskiy, Witkowski & Pliński, *Cocconeis euglypta* Ehrenberg, *H. normanii* (Rabenhorst) Levkov, *N. inconspicua* and *Rhopalodia operculata* (C. Agardh) Håkansson (6 of the 8 springs).

A floristic list of all taxa with a minimum RA >1% in all samples and their ecological preferences is reported in Table 3. These species are mainly linked to the aquatic environment, with preference for alkaline and fresh-brackish waters and high nutrient contents. The most abundant and common species at several sites in each region were illustrated by scanning electron microscopy (Fig. 3A-H).

The results of the biotic integrity indices are reported in Table 4. The minimum and maximum values of species richness, Shannon-Wiener index and Pielou index (evenness) were found respectively at LEFO and BARD2 from Auvergne and at SMA and CAD 2 from Sardinia.

Table 3. Floristic list of all diatom taxa with relative abundance >1% , relative abundances and ecological preferences for pH, salinity, trophic state and moisture according to Van Dam et al. (1994). Preferences for **pH**: ak = alkaliphilous, n = circumneutral; preferences for **Salinity** (S): f-b = fresh brackish, b = brackish; b-f = brackish-fresh; preferences for **Trophic State** (TS) (species characteristic of): oligo = oligotrophic environments, eu = eutrophic environments; hyper = hypereutrophic environments; preferences for **Moisture** (M): 1= never, or only very rarely, occurring outside water bodies, 2 = mainly occurring in water bodies, sometimes on wet places, 3 = mainly occurring in water bodies, also rather regularly on wet and moist places, 4 = mainly occurring on wet and moist or temporarily dry places, 5 = nearly exclusively occurring outside water bodies.

		Auvergne-France								**Sardinia-Italy**											
Taxa	Sigles	PSAL	FDBL	BARD2	LEFO	CERI	CHAT	BENE	POIX1	SGA	CAD 1	CAD 2	SSA	ABB	SBM	SMA	CAS	pH	S	TS	M
Achnanthidium exiguum (Grunow) Czarnecki	ADEG									0.6	0.4	0.9	2.0	0.0	44.6	0.0	0.1	ak	f-b	oligo-eu	3
Achnanthidium minutissimum (Kützing) Czarnecki	ADMI									1.1	0.5	2.4	0.9	0.0	3.3	0.0	0.0	n	f-b	hyper	3
Aulacoseira granulata (Ehrenberg) Simonsen	AUGR									0.0	0.6	0.0	0.3	0.0	0.0	0.0	19.2	ak	f-b	eu	1
Cocconeis euglypta Ehrenberg	CEUG									0.1	3.1	4.2	2.2	0.0	0.2	0.0	0.6	ak	f-b	eu	2
Crenotia thermalis (Rabenhorst) Wojtal	CRTH	68.2	0.2	49.9	96.2	45.8	96.8	38.3	0.0	0.0	0.0	0.0	0.0	4.5	0.1	6.3	0.0	n	b-f		
Encyonema silesiacum (Bleisch) D.G. Mann	ESIL									0.0	0.0	0.0	25.9	0.0	0.0	0.0	0.0	ak	f-b	hyper	1
Fallacia pygmea	FPYG	0.0	0.0	3.1	0.0	0.0	0.0	0.0	0.0												
Fallacia sp.	FALS	0.0	0.0	1.2	0.0	0.0	0.0	0.0	0.0												
Navicula gregaria Donkin	NGRE	0.0	0.0	0.0	0.0	0.0	0.0	0.0	29.6									ak	b-f	eu	3
Navicula salinarum Grunow	NSAL	0.0	0.0	0.0	0.0	0.0	0.0	0.0	9.0									n	b	eu	1
Navicula sanctamargaritae A. Beauger	NYSG	30.0	0.0	12.5	0.0	30.3	0.0	55.4	0.0	0.0	0.0	0.0	0.0	20.4	0.0	34.5	0.0				
Navicula veneta Kützing	NVEN	0.0	0.0	1.2	0.0	1.2	0.2	0.0	6.3	0.4	1.0	1.3	4.5	4.6	0.9	2.7	0.2	ak	f-b	eu	3
Navicula vilaplanii (Lange-Bertalot & Sabater) Lange-Bertalot & Sabater	NVIP																				
Nitzschia aff. *bulnheimiana*	N. aff. bul	0.0	0.0	0.0	0.0	0.0	0.0	0.0	18.0												
Nitzschia amphibia Grunow	NAMP									19.0	2.3	7.2	0.2	0.1	1.0	0.0	0.2	ak	b	eu	1
Nitzschia aff. *liebetrhutii*	N. aff. lieb									0.0	0.0	0.0	0.0	0.0	0.0	0.0	7.2				
Nitzschia inconspicua Grunow	NINC									1.7	8.9	2.3	1.1	1.7	8.1	0.0	0.0	ak	b-f	eu	3
Nitzschia microcephala Grunow in Cleve & Möller	NMIC									0.0	1.5	0.5	3.2	0.0	2.3	0.0	1.5	ak	f-b	eu	1
Nitzschia palea (Kützing) W. Smith	NPAL									0.0	19.3	0.5	0.4	0.0	0.9	0.0	0.5	n	f-b	hyper	3
Nitzschia supralitorea Lange-Bertalot	NZSU	0.0	0.0	3.1	0.0	0.0	0.0	0.0	0.0												
Pinnularia kuetzingii Krammer	PKUT	0.0	0.0	0.5	3.5	0.0	1.7	0.0	0.0												
Pinnularia sp.3	PINS									0.0	0.0	0.0	0.0	9.4	0.0	33.8	0.0				
Planothidium frequentissimum (Lange-Bertalot) Lange-Bertalot	PLFR	0.0	1.4	10.3	0.0	21.7	0.2	4.4	2.4	0.5	0.0	0.0	6.0	32.7	2.2	0.0	0.0	ak	f-b	oligo-eu	
Planothidium lanceolatum (Brébisson ex Kützing) Lange-Bertalot	PTLA	0.0	61.1	0.0	0.0	0.0	0.0	0.0	0.0									ak	f-b	eu	3
Planothidium victorii P.M. Novis, J. Braidwood & C. Kilroy	PVIC	0.0	0.0	0.0	0.0	0.2	0.0	1.9	0.0												
Pseudostaurosira bardii Beauger, C.E. Wetzel & Ector	PBAR	0.0	0.0	3.4	0.0	0.0	0.0	0.0	0.0												
Pseudostaurosira brevistriata (Grunow) D.M. Williams & Round	SBRE									0.0	0.6	5.6	0.0	0.0	0.0	0.0	1.2	ak	f-b	oligo-eu	2
Rhopalodia operculata (C. Agardh) Håkansson	ROPE									0.0	0.9	0.6	1.9	5.4	0.0	5.4	3.1				
Sellaphora labernardierei A. Beauger, C.E.Wetzel & L. Ector	SLAB	0.0	31.5	0.7	0.0	0.0	0.0	0.0	0.0												
Sellaphora nigri (De Notaris) C.E. Wetzel & L. Ector	SNIG	0.0	1.0	7.0	0.0	0.0	0.0	0.0	0.0	52.8	0.2	1.5	1.4	0.0	0.0	0.0	0.0				
Sellaphora saugerresii (Desmazières) C.E. Wetzel & D.G. Mann	SSGE									4.1	0.0	0.0	0.0	8.3	0.0	0.0	0.0				
Stephanodiscus neoastraea Håkansson & Hickel	SNEO									0.0	3.7	0.8	0.0	0.0	0.0	0.0	14.8	akb	f-b	eu	1
Tryblionella apiculata Gregory	TAPI	0.0	0.0	0.0	0.0	0.0	0.0	0.0	12.1												
Tryblionella hungarica (Grunow) D.G. Mann	THUN	0.0	0.0	0.0	0.0	0.0	0.0	0.0	13.6									ak	b-f	eu	1
Tryblionella sp.	TRYS	0.0	0.0	0.0	0.0	0.0	0.0	0.0	3.2												
Ulnaria ulna (Nitzsch) Compère	UULN									1.5	3.1	1.8	0.0	0.0	0.1	0.0	0.8	ak	f-b	oligo-eu	1

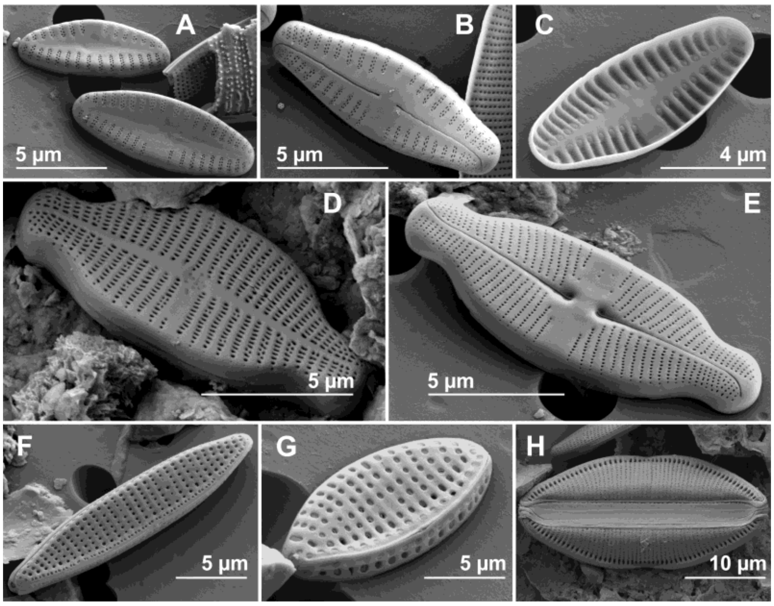

Fig. 3. Scanning electron microscopy (SEM). *Crenotia thermalis*, external rapheless valve view (A), external raphe valve view (B), internal rapheless valve view (C); *Lemnicola exigua*, external rapheless valve view (D), external raphe valve view (E); *Nitzschia amphibia*, external valve view (F); *Nitzschia inconspicua*, external valve and mantle view (G); *Rhopalodia operculata*, external frustule view.

Table 4. Values of species richness, Shannon-Wiener diversity index (H') and Pielou evenness index (J') for diatom assemblages of Auvergne and Sardinia.

	RS	**H'**	**J**
Auvergne-France			
PSAL	8	1.05	0.35
FDBL	15	1.36	0.35
BARD2	25	2.70	0.58
LEFO	3	0.24	0.15
CERI	8	1.68	0.56
CHAT	7	0.27	0.09
BENE	4	1.31	0.65
POIX1	20	3.00	0.69
Sardinia-Italy			
SGA	26	2.48	0.53
CAD1	52	4.28	0.75
CAD2	72	5.22	0.85
SSA	38	3.45	0.66
ABB	15	2.44	0.64
SBM	35	2.79	0.56
SMA	6	0.93	0.38
CAS	43	3.84	0.71

Table 5. Synthesis of results for the SIMPER analysis (taxa which contributed to the differences among diatom assemblages between Auvergne and Sardinia and among diatom within each geographic region with their percentage contribution at the cut-off level of 70%).

Auvergne vs Sardinia (average dissimilarity 93.49%)		
Taxons	Contribution%	Contribution cumulative %
Crenotia thermalis	33.3	33.3
Navicula sanctamargaritae	11.6	45.0
Planothidium lanceolatum	5.3	50.3
Planothidium frequentissimum	5.2	55.4
Sellaphora nigri	4.7	60.1
Lemnicola exigua	4.1	64.2
Pinnularia sp. 3	3.2	67.4
Sellaphora labernardierei	2.8	70.2
Auvergne (average dissimilarity 33.98%)		
Taxon	Contribution%	Contribution cumulative %
Crenotia thermalis	81.6	81.6
Sardinia (average dissimilarity 13.01%)		
Taxons	Contribution%	Contribution cumulative %
Nitzschia inconspicua	14.9	14.9
Navicula veneta	11.8	26.7
Rhopalodia operculata	10.3	37.0
Nitzschia amphibia	8.8	45.8
Cocconeis euglypta	6.7	52.5
Navicula sanctamargaritae	6.6	59.8
Nitzschia microcephala	6.5	65.5
Achnanthidium minutissimum	4.8	70.3

Differences between assemblages and relationships with environmental variables

The diatom assemblages from springs of Auvergne and Sardinia were clearly separated in the nMDS ordination plot (Fig. 4). Significant differences between the two groups were confirmed by the ANOSIM test (global R = 0.516; p = 0.2%). According to the SIMPER analysis the average dissimilarity was 93.49%. The greatest contribution to the differences was provided by *Crenotia thermalis* (33.34%) and *Navicula sanctamargaritae* (11.64%) (Table 5). The assemblages from Auvergne revealed a higher average similarity among sites (33.98%) than assemblages from Sardinia (13.01%).

The greatest contribution to the differences was provided by *C. thermalis* (81.63%) for the sites of Auvergne and *Nitzschia inconspicua* (14.86%), *Navicula veneta* (11.81%) and *Rhopalodia operculata* (10.33%) for the sites of Sardinia (Table 5).

The CCA analysis (Fig. 5 and Fig. 6) explained 81.7% of the variance in the data in the 2 first axes (axis 1: 28.2% and axis 2: 53.5%). Ordination data distinguished 3 main groups of species: 1) species like *Achnanthidium minutissimum* (Kützing) Czarnecki, *Cocconeis euglypta*, *Encyonema silesiacum* (Bleisch) D.G. Mann, *Lemnicola exigua* and *Nitzschia microcephala* from several springs of Sardinia associated with higher values of pH; 2) species like *C. thermalis*, *Navicula gregaria* Donkin, *N. salinarum* Grunow, *Nitzschia* aff. *liebethruthii*, *Pinnularia kuetzingii* Krammer, *Tryblionella apiculata* W. Gregory and *T. hungarica* from most of the springs of Auvergne and ABB and SMA of Sardinia, associated with higher values of NH_4^+, conductivity and HCO_3^-; 3) *Planothidium lanceolatum* (Brébisson ex Kützing) Lange-Bertalot and *Sellaphora labernardierei* Beauger, C.E. Wetzel & Ector from LEFO in Auvergne associated with higher values of $N\text{-}NO_3^-$.

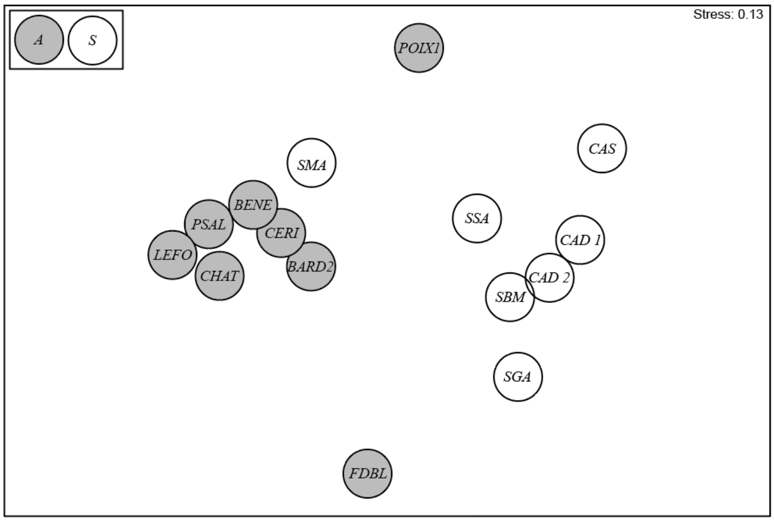

Fig. 4. nMDS ordination plot for springs of Auvergne and Sardinia. Gray circles = sites of Auvergne; white circles = sites of Sardinia. All sigles of sites are reported in Table 1.

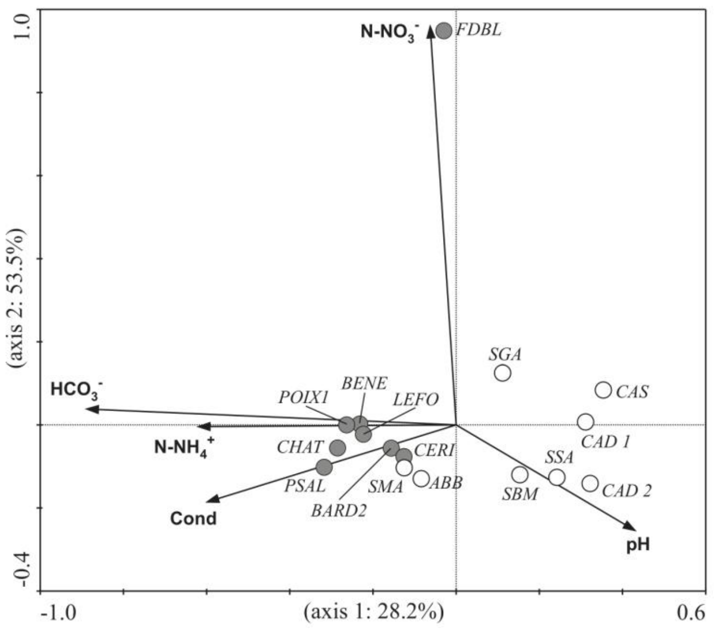

Fig. 5. CCA ordination plot for sites. Vectors = environmental variables; gray circles = sites of Auvergne; white circles = sites of Sardinia.

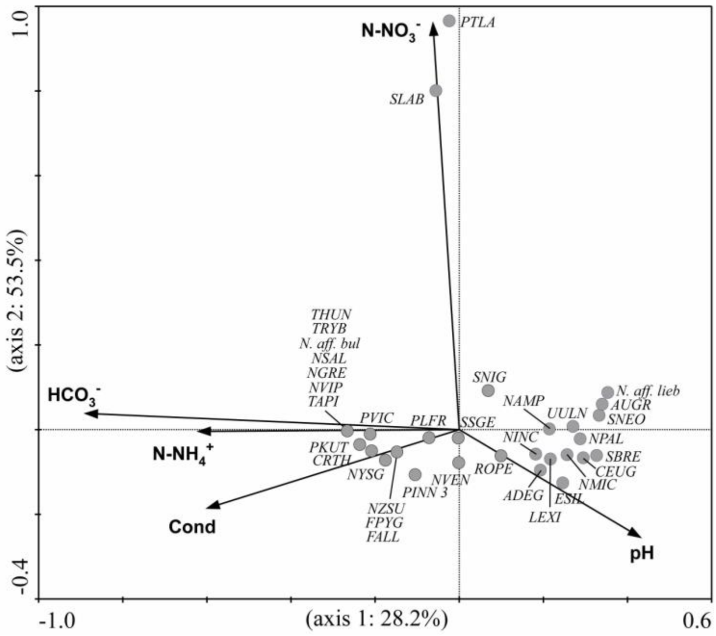

Fig. 6. CCA ordination plot for diatom species. Vectors = environmental variables; circles = diatom species. All sigles of species are reported in Table 3.

Discussion

Structure of the diatom assemblages

The diatom assemblages from thermo-mineral springs of Auvergne and Sardinia showed a high level of biodiversity. Diatoms were found in all investigated sites, including those with high water temperatures and mineralization, factors generally considered not very conducive for diatom growth (Mandal & Sarkar, 2015). Our observations are supported by studies made in different geographic areas, such as Africa (Mpawenayo, Cocquyt & Nindorera, 2005), Azores (Quintela et al., 2013), Czech Republic (Kaštovský & Komárek 2001), France (Beauger et al., 2017), Italy (Andreoli & Rascio, 1975), Russia (Nikulina & Kociolek 2011) and Thailand (Pumas et al., 2018) that reported diatom assemblages at temperatures >50°C. Several studies also described diatom assemblages in springs with high mineral content (conductivity from 4580 to 18340 μS cm^{-1}) (Mpawenayo, Cocquyt & Nindorera, 2005; Beauger et al., 2017; Angel et al., 2018).

In general, in Auvergne, species richness, diversity and evenness were higher in springs with lower temperature (FDBL, POIX1 and BARD2) and lower in springs with higher mineralization (PSAL and CHAT). In Sardinia, the values of these indices were higher in warmer springs (CAD1 and CAD2) and, as in Auvergne, lower in more mineralized springs (SMA and ABB). Our results, although partially in contrast, are in good agreement with those reported in literature. For example, in springs of Galicia in Spain, the species richness was higher in hot springs than cold springs and only seven taxa were found in a cold-water spring with moderate mineralization (Leira, Meijide-Failde & Torres, 2017). Furthermore, in thermo-mineral springs from Azores, the highest values of diversity were recorded in two sites, respectively cold and thermal (17-58 °C) (Quintela et al. 2013). This suggests that different combinations of water temperature and mineralization can support assemblages differently structured. The values of the indices observed at CAS, although unusually high, were lower than the other two hottest springs CAD1 and CAD2. This seem in accordance with the results of other studies that reported an impoverishment of species at temperatures >70°C (e.g. Nikulina & Kociolek, 2011; Quintela et al., 2013). A previous study at CAS also showed a lower species richness in asseblages collected close to the water emergence point, where the temperature reaches 70 °C (Lai et al., 2018).

The high number of species found in this study suggests wide spatial heterogeneity and variability of environmental conditions. In fact, these factors generate high habitat diversity and high total species richness in springs according to Cantonati et al. (2012). In Sardinia the high geodiversity (Fiorentino, Curioni & Pisano, 2017) probably plays an important role in the richness of diatom flora.

Species composition in the two geographical regions

In Auvergne, the thermo-mineral springs sheltered 61 different taxa and were characterized by a large heterogeneity among sites with some species occuring only once. At BARD2, the presence of *Pseudostaurosira bardii*, recently described from this site is interesting (Beauger et al., 2018). This spring is slightly acidic pH (6.53) with an elevated conductivity level (6510 µS cm^{-1}) and is enriched with sodium-chloride and bicarbonate. Moreover, *Sellaphora labernardierei* was also present.

This last species was recently described at FDBL. This spring is slightly acidic pH (6.4), with a medium conductivity level (1344 µS cm–1) and is enriched with calcium (236.91 mg L^{-1}) and nitrates (Beauger et al., 2016). On the contrary, *Crenotia thermalis* was observed in all springs (except POIX1) and in LEFO, characterized by the highest water temperature (32.6°C) and reached RA = 96%. This species, occasionally encountered in running waters, is characteristic of electrolyte-rich inland habitats, particularly thermal and mineral springs (Lange-Bertalot, et al. 2017). Among the *Navicula*, *N. sanctamargaritae* was observed in PSAL, BARD2, CERI and BENE in association with *C. thermalis* mainly. This taxon was first described in the Massif Central, in a thermo-mineral spring highly mineralized (Beauger et al., 2015). *Navicula veneta*, a cosmopolitan species, known to live in electrolyte-rich to brackish waters (Lange-Bertalot, 2001), was present at BARD2, CERI, CHAT and POIX1. This species is present in other thermo-mineral springs of the world as in Spain (Leira, Meijide-Failde & Torres, 2017) and Chile (Angel et al., 2018). The spring POIX1 presented a particular community dominated by *N. gregaria*, *N. salinarum*, *N. veneta*, *Nitzschia* aff *bulnheimiana* and different *Tryblionella* species. This spring is characterized by degassing of hydrogen sulphide. *N. gregaria* and *N. salinarum* are present respectively on coasts and inland salt springs, and in inland brackish waters (Lange-Bertalot, 2001).

Navicula salinarum is not classically encountered in springs contrary to *N. gregaria* observed in different countries (Owen, Renaut & Jones, 2008; Leira, Meijide-Failde & Torres, 2017; Angel et al., 2018). *Tryblionella apiculata* and *T. hungarica* are observed in salt-rich inland habitats (Lange-Bertalot et al., 2017), as in Chile, where they are associated with freshwater fumeroles (Angel et al., 2018). No centrics diatom species were observed in the springs studied. In Sardinia the heterogeneity among sites was higher than Auvergne and several species were found at only one site. The most abundant species included *L. exigua*, *Encyonema silesiacum*, *N. sanctamargaritae*, *Pinnularia* sp.3, *P. frequentissimum* and *Sellaphora nigri* (De Notaris) C.E. Wetzel & Ector. *Lemnicola exigua* was found in geothermal springs of several geographic areas (e.g. López-Sandoval et al., 2016; Angel et al., 2018; Pumas, Pruetiworanan & Peerapornpisal, 2018). *Encyonema silesiacum* was reported in Sakhalin springs in Russia (Nikulina & Kociolek, 2011). *Navicula sanctamargaritae*, *P. frequentissimum* and *S. nigri* were found in springs of the French Massif Central region (e.g. Beauger et al., 2016; Beauger et al., 2017). According to Van Dam, Mertens & Sinkeldam (1994), *L. exigua* and *P. frequentissimum* have a wide ecological range. In our study *L. exigua* was common at 6 sites but was not observed in the two cooler and salt springs (ABB and SMA). *Planothidium frequentissimum* was common at 4 sites and was not found in the sites with high temperature (CAD1, CAD2 and SMA) and with low temperature and high conductivity (SMA). *Encyonema silesiacum* is a species α-mesosaprobic and hypereutraphentic according to Van Dam, Mertens & Sinkeldam (1994). By contrast, it is considered characteristic of little disturbed habitats from human activities but tolerant to nutrient enrichment and prefers low-medium electrolyte content according to Lange-Bertalot et al. (2017). In our study this species was observed only at SSA spring, characterized by low content of nitrates and medium-high conductivity. This is in accordance with Nikulina & Kociolek (2011) who reported *E. silesiacum* as a species indifferent to salinity. *Sellaphora nigri* is also a species with a wide ecological range, generally more abundant in degraded habitats according to Lange-Bertalot et al. (2017). In our study this species was common at 3 sites but was more abundant at SGA spring, characterized by the higher concentration of nitrates. *Navicula sanctamargaritae*, also observed in several springs of Auvergne, was found only in 2 sites of Sardinia, ABB and SMA, with very similar environmental characteristics to the French sites.

Crenotia thermalis was also abundant in these sites. Other abundant species included *Nitzschia amphibia*, *Nitzschia* aff. *liebethruthii*, *N. inconspicua*, *N. palea* (Kützing) W. Smith, *Pseudostaurosira brevistriata* (Grunow) D.M. Williams, *Rhopalodia operculata* and *Sellaphora saugerresii* (Desmazières) C.E. Wetzel & D.G. Mann. Most of these species are reported in thermo-mineral systems by several authors (e.g. Villeneuve & Pienitz, 1998; Mpawenayo & Mathooko, 2004; Mannino, 2007; Quintela et al., 2013; Bhakta, Das & Adhikary, 2016; Angel et al., 2018). Centric diatoms like *Aulacoseira granulata* and *Stephanodiscus neoastraea* were found respectively at CAD1, SSA and CAS, and at CAD1, CAD2 and CAS, and were abundant at CAS. The higher abundance in this later spring is consistent with the high mineralization level of water, because this species mostly prefers fresh-brackish waters according to Van Dam, Mertens & Sinkeldam (1994).

Comparison among diatom assemblages

The diatom assemblages showed a high dissimilarity level both within each geographic region and between Auvergne and Sardinia. In Auvergne significant differences were observed especially for the FDBL and POIX1 springs which resulted well separated between them and from a cluster formed by the remaining six sites. The FDBL spring was dominated by *Planothidium lanceolatum* and *Sellaphora labernardierei*. The POIX1 spring was dominated by *Navicula gregaria* and *Nitzschia* aff. *bulnheimiana*. In addition, *Crenotia thermalis* dominant in all 6 springs forming the cluster, was not found at POIX1 and only one specimen was observed at FDBL. In Sardinia, CAD1, CAD2 and SBM springs formed a separate cluster from the remaining five springs which were different from each other. These three springs showed several common species with quite similar abundances, except for *Lemnicola exigua*, most abundant at SBM and *Nitzschia palea* most abundant at CAD1. Overall, the springs of Sardinia showed a higher species richness, including many rare taxa (RA ≤ 1%) compared to the springs of Auvergne. Consequently, the assemblages of the two regions formed two clearly separated clusters. The differences were due to several species but a greater contribution was provided by *C. thermalis* and *Navicula sanctamargaritae*, widely distributed in springs of Auvergne and found only in two springs of Sardinia (ABB and SMA). These results highlighted a wide spatial heterogeneity in distribution of species.

This variability is not surprising since several studies reported different richness and species composition patterns among thermo-mineral systems both on a local and regional scale (e.g. Mpawenayo & Mathooko, 2004; Owen et al., 2004; Owen, Renaut & Jones, 2008). Local factors such as water temperature, light, substratum particle size, current velocity (discharge) and water chemistry are considered important drivers for growth and distribution diatom species (Patrick, 1948; Cantonati, Gerecke & Bertuzzi, 2006). For example, observations on diatom flora from many cold springs of Sardinia highlighted the importance of the hydrogeological conditions in the variations of the species composition among sites (Lange-Bertalot et al., 2003). In addition, diatom assemblages can also vary considerably in different geographic regions in relation to the dispersal mechanisms of species and variations of climate-related factors (solar radiation, cloudiness, temperature, and seasonality) (Owen, Renaut & Jones, 2008; Vanormelingen, Verleyen·& Vyverman, 2008).

Relationships of diatoms with environmental variables

Among the environmental variables measured and analyzed in this study, pH, conductivity, HCO_3^-, $N\text{-}NH_4^+$ and $N\text{-}NO_3^-$ explained significant amount of variance in diatom assemblages according to the CCA analysis. Most of these variables, especially pH and conductivity, are reported as significant for diatom assemblages in several geothermal systems (e.g. Owen et al., 2004; Mpawenayo, Cocquyt & Nindorera, 2005; Owen, Renaut & Jones, 2008; Angel et al., 2018; Pumas, Pruetiworanan & Peerapornpisal, 2018). In addition, the total ion concentration and nutrients play a very important part in the distribution of diatoms according to several authors (Cantonati, Gerecke & Bertuzzi, 2006; Soininen, 2007; Wojtal & Sobczyk, 2012). The clusters of species reflected differences in the physical and chemical characteristics of the studied springs in a consistent way with the PCA analysis based on environmental variables.

Conclusions

The springs studied showed high species richness, especially in Sardinia, although winter is not the most favourable season for growth and diversity of diatom flora. Significant differences were found among diatom assemblages both within each geographic region and between Auvergne and Sardinia, according to our initial hypothesis.

The differences observed among sites within each region, probably due to the combined influence of several local factors, did not allow us to establish characteristic combinations of species for these environments, similarly to other studies. In addition, several taxa have broad ranges of tolerance to the major environmental variables and are also found in no thermo-mineral systems. However, some taxa can be considered more representative for springs of each region based on their greater abundance and the common occurrence in multiple sites: 1)

Crenotia thermalis for Auvergne 2) *Lemnicola exigua*, *Nitzschia amphibia*, *Nitzschia inconspicua* and *Rhopalodia operculata* for Sardinia. The level of dissimilarity found between the two geographic regions, unexpectedly very high, suggests that also climatic factors can be an important driver for the species distribution. Among the environmental variables tested, pH, conductivity, N-NH_4^+, N-NO_3^-, and HCO_3^- were the most significant for species distribution.

The present work provides a first framework on diversity, ecology and distribution of diatoms in thermo-mineral springs of different geothermal settings of Auvergne and Sardinia. The results obtained underline the high heterogeneity of these spring environments also from the biological point and the presence of a partly still unknown biodiversity. Further studies focused on taxonomic aspects could allow to define the identity of some abundant and sometimes dominant taxa but not yet identified at specific level in this study. Their identification is a crucial step for ecological studies in similar environments and their comparison.

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7. Chapter III

Accepted in Oceanological and Hydrobiological Studies

Submissions with an Editorial Office Decision for Author Giuseppina Grazia Grazia Lai, PhD Student

Page: 1 of 1 (1 total completed submissions) Display 10 results per page.

Action	Manuscript Number	Title	Initial Date Submitted	Status Date	Current Status	Date Final Disposition Set	Final Disposition
View Submission View Decision Letter Send E-mail	OandHS-D-18-00042	Chamaepinnularia thermophila (Bacillariophyceae): synonymy with Navicula tongatensis Hustedt and update of its geographic distribution and ecology	11-09-2018	26-10-2018	Completed Accept	26-10-2018	Accept

Page: 1 of 1 (1 total completed submissions) Display 10 results per page.

Chamaepinnularia thermophila (Bacillariophyceae): synonymy with *Navicula tongatensis* Hustedt and update of its geographic distribution and ecology

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Running Title: An update on *Chamaepinnularia thermophila*

Keywords: *Chamaepinnularia tongatensis*, *Navicula thermophila*, species distribution, ecology, springs, thermo-mineral waters

Abstract

Chamaepinnularia thermophila is a small and poorly known diatom species. After the first description from a hot spring in Guadalupe in 1952, its presence seemed limited to a few other springs of the French Antilles. The aim of this study is to report new information on aspects concerning taxonomy, distribution and ecology of this species. Accurate analysis by light and scanning electron microscope of material collected on different substrates (cobbles, macrophytes and fine sediments) from a thermo-mineral spring of Sardinia (Italy), allowed us to document the first record of the species in the Mediterranean area. Moreover, the comparison with *Navicula tongatensis* from Hustedt's original material, carried out because of their similarity, revealed identical morphological characteristics suggesting their consequent synonymy. Based on the information available in the literature and our data, *C. thermophila* is a rare species mainly present in tropical areas, in thermal springs with alkaline pH, medium-high conductivity and low-moderate nutrient content. The occurrence of the species in sites with very different environmental characteristics seems unusual but it could indicate a broader ecological range. This study contributes to uniform the nomenclature used for this species so far, and provides a first framework on its global geographic distribution and ecology.

Introduction

The genus *Chamaepinnularia* was originally described by Lange-Bertalot & Krammer (Lange-Bertalot & Metzeltin 1996) and comprises isolated and relatively small cells (up to 25 µm in length and 4 µm in width). Slightly larger dimensions (up to 30 µm in length and 4.9 µm in width) were reported for *Chamaepinnularia gerlachei* from Antarctica (Van de Vijver et al. 2010). Representatives of the genus have linear-elliptic to linear-lanceolate valve outlines, sometimes sinuous, with rounded apices. The striae, arranged in single row, are composed by large areolae interrupted by a silica line near the valve face-mantle junction along the apical plane. The openings of the areolae are covered by vela externally and divided with plates or silica bridges internally (Lange-Bertalot & Metzeltin 1996; Wetzel et al. 2013).

According to Kociolek et al. (2018), the genus currently contains 63 taxa of which 20 uncertain, including several species previously allocated to the genera *Navicula* or *Pinnularia* by different authors (e.g. Petersen 1915, 1928; Krasske 1929; Hustedt 1934, 1942).

The genus *Chamaepinnularia* has a cosmopolitan distribution and is often an important component of diatom assemblages in pristine areas and habitats less affected by anthropogenic activities, including springs (Cantonati & Lange-Bertalot 2009; Wetzel et al. 2013). Several species are typical of aerial habitats (on/around wet mosses and soil) (Metzeltin & Lange-Bertalot 1998, 2007; Wydrzycka & Lange-Bertalot 2001; Cavacini et al. 2006; Cocquyt 2007; Van de Vijver et al. 2010; Van de Vijver & Cox 2013; Żelazna-Wieczorek & Olszyński 2016). However, different species occur in fresh waters, eg.: *C. amphiborealis* Lange-Bertalot & Werum in Werum & Lange-Bertalot, *C. begeri* (Krasske) Lange-Bertalot in Lange-Bertalot & Metzeltin, *C. schauppiana* Lange-Bertalot & Metzeltin and *C. vyvermanii* Lange-Bertalot in Lange-Bertalot & Metzeltin, in fresh to brackish waters, eg.: *C. krookii* (Grunow) Lange-Bertalot & Krammer apud Lange-Bertalot & Genkal and *C. krookiformis* (Krammer) Lange-Bertalot & Krammer apud Lange-Bertalot & Genkal and in brackish waters, e.g.: *C. gibsonii* Van de Vijver in Van de Vijver et al. Few *Chamaepinnularia* species are also found in marine environments, eg.: *C. clamans* (Hustedt) Witkowski, Lange-Bertalot & Metzeltin and *C. truncata* (D. König) Witkowski, Lange-Bertalot & Metzeltin (Witkowski et al. 2000; Wetzel et al. 2013; Żelazna-Wieczorek & Olszyński 2016).

Chamaepinnularia thermophila (Manguin) C.E.Wetzel & Ector was originally described as *Navicula thermophila* by Manguin in 1952, from a hot saline spring in Guadalupe in the French Antilles (Bourrelly & Manguin 1952). In 2007, this species was excluded with certainty by *Navicula sensu stricto* and was provisionally named *Naviculadicta thermophila* by Metzeltin & Lange-Bertalot (2007), until a definition of a more appropriate genus. Finally, in 2016, the species was transferred to the genus *Chamaepinnularia* on the basis of further structural analyses in light (LM) and scanning electron microscopy (SEM) of the original material of Manguin (Wetzel & Ector 2016). *Chamaepinnularia thermophila* is a small and poorly known species, rarely recorded in Europe and other continents.

The available information on this species are scattered and not comprehensive so far. In some cases, the specific location and characteristics of the occurrence sites as well as the ecology of the species are not clearly defined in literature.

The aims of this study were: 1) to highlight the synonymy of *C. thermophila* and *Navicula tongatensis* Hustedt by LM and SEM comparison; 2) to give additional information and data after the first record in Sardinia (Italy); 3) to provide a first framework on the global geographic distribution and ecology of the species.

Material and methods

Study site

The spring San Giovanni Su Anzu (Fig. 1) is located in the eastern extremity of the Monte S’Ospile, in the Supramonte massif (central-eastern Sardinia) (Mucedda & Fancello 2002). The territory is characterized by Paleozoic granites and metamorphic rocks, limestones and dolostones of Middle Jurassic and Upper Cretaceous, and Mesozoic carbonate outcrops (De Waele & Grafitti 2004). The spring (40°19'15.3" N, 009°37'05.6"E) is a small rheocrenic system that flows at about 151 m a.s.l. The water is partly collected in a tub, locally named Lapia, inside a small covered structure, and is used for free thermal baths by the local community and tourists (Fiorentino et al. 2017). Part of the water form a rivulet that flows in karst waters coming from the nearby cave San Giovanni Su Anzu-Ispinigoli, at short distance from the emergence point. The water, known since Roman times, is hypothermal, with a bicarbonate-alkaline-earth chemistry (Bacciu 2009).

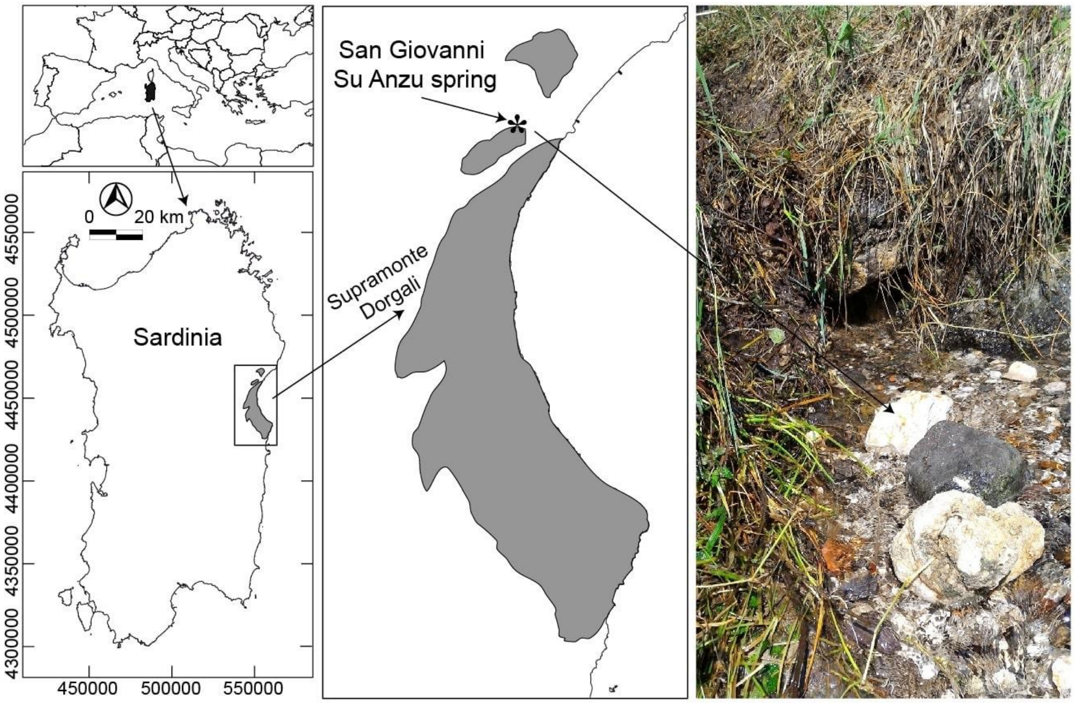

Fig. 1: Geographic location and photo of the San Giovanni Su Anzu thermo-mineral spring (Sardinia, Italy).

Sampling and analyses

Water and diatom samples were collected simultaneously in July 2016 and February 2017 in the spring-fed rivulet. The water temperature, pH and conductivity were measured *in situ* with a multiparameter probe (YSI ProPlus). Repeated measurements of discharge (Q) and water depth were made respectively by the volumetric method using a 1 l bottle and a chronometer and a graduated metric rod.

The water samples were collected using 1 l polyethylene bottles. The samples for dissolved oxygen were collected in 150 ml glass bottles and immediately fixed *in situ* for the laboratory analyses with the Winkler method (Winkler 1888). Biochemical Oxygen Demand (BOD_5) was determined by direct titration of the samples after aeration up to saturation and incubation for five days at a temperature of 20 °C and dark conditions. Physical and chemical analyses, including ions and trace elements, were carried out following standard methods (Strickland & Parsons 1972; APAT/IRSA & CNR 2003).

Diatom samples were collected from three different substrates (cobbles, macrophytes and fine sediments), following the methods reported in Kelly et al. (1998) and ISPRA (2014). The epilithic diatoms were collected from five cobbles randomly selected with a hard-bristled toothbrush. Epiphytic diatoms were collected from submerged portions of *Mentha insularis* Req., *Lotus rectus* L. and *Typha* cf. *domingensis* (Pers.) Steudel. The material collected from macrophytes was integrated into a composite sample. The epipelic diatoms were collected from fine surface sediments by glass tubes. All diatom samples were immediately fixed with formaldehyde to a final concentration of 4%.

Diatom subsamples were treated with hydrogen peroxide (30% v/v) on a heating plate. Diluted hydrochloric acid (37% v/v) was added on the cooled material to remove carbonates, according to ISPRA (2014).

LM observations, measurements and counts were done on slides mounted by Styrax® resin (refractive index = 1.59) using a Leica® DMR microscope with 100x oil immersion objective. Count data were converted into percentage relative abundances (RA) in respect to the total (~ 400 valves) for each sample. Micrographs were taken with a Leica® DFC 500 high-resolution digital camera using Leica Application Suite software (v. 3.7.0, Leica Microsystems©).

For SEM observations, subsamples on aluminum stubs sputtered with platinum were observed with a Hitachi SU-70 field emission scanning electron microscope.

The Hustedt's original material of *Navicula tongatensis* (Hustedt collection, material number AT341, corresponding to the lectotype slide number 345/57. Nuku'alofa, Tonga-Ins., aL 9. Well. Specimen ID H46586, year 1954) was used for LM and SEM comparison.

LM and SEM images were manipulated using CorelDraw X6.

Results

Chamaepinnularia thermophila (Manguin) C.E.Wetzel & Ector 2016

Basionym: ≡ *Navicula thermophila* Manguin in Bourrelly & Manguin 1952

Synonyms: = *Navicula tongatensis* Hustedt 1962; = *Chamaepinnularia tongatensis* (Hustedt) Lange-Bertalot in Lange-Bertalot & Metzeltin 1996; ≡ *Naviculadicta thermophila* Metzeltin & Lange-Bertalot 2007.

The main characteristics and the type locality of *N. thermophila* and *N. tongatensis* reported in the original descriptions are summarized in Table 1.

Table 1. Main characteristics and Type locality of *Navicula thermophila* and *Navicula tongatensis* reported in the original descriptions of the species.

	Navicula thermophila	=	*Navicula tongatensis* Hustedt
Valve outline	elliptic		linear-elliptic
Ends	capitate		sub-capitate
Raphe			straight, filiform
Striae	radiate, more spaced in the middle		slightly radiate, more spaced in the middle
Central area	sub-quadratic		
Length (μm)	7.2-8.0		6.0-8.0
Width (μm)	2.5-3.0		2.5
Striae/10 μm	29-30		26-28
Type Locality	hot - salt spring		thermal well

Specimens from the San Giovanni Su Anzu spring (Sardinia): main morphological characteristics

The specimens observed show ends subcapitate and rounded. The striae, slightly radiate, are more widely spaced in the centre of the valve but difficult to resolve with LM. The central area is very small. The raphe is filiform and straight with distal ends bent towards the same side and a central silica nodule is present. They are composed of three large areolae interrupted by the silica deposition along the apical plane clearly visible with SEM.

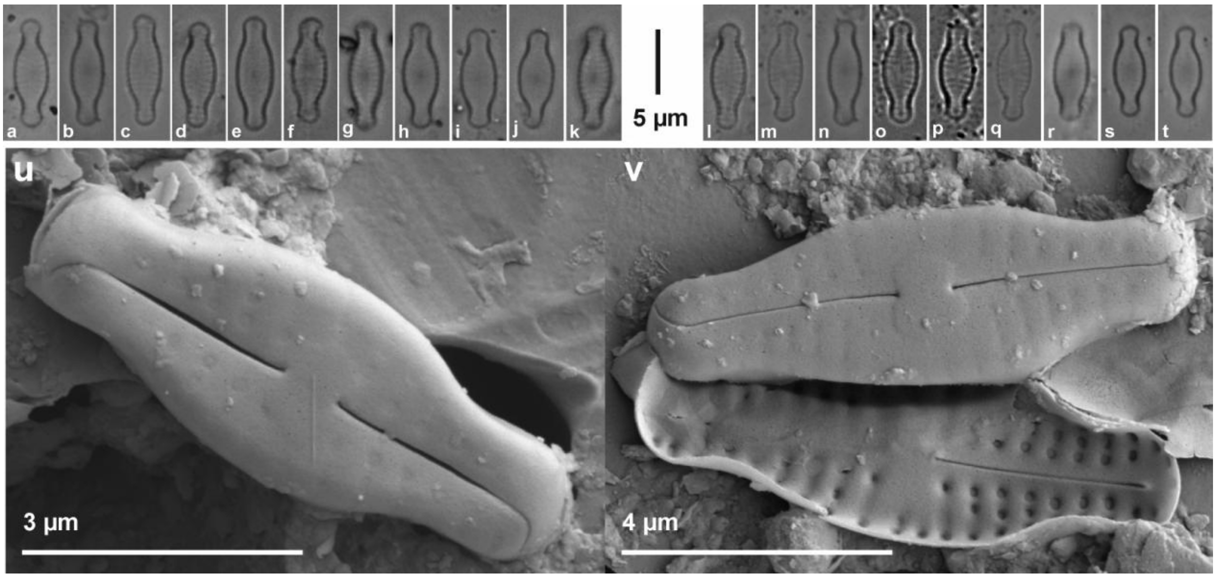

Fig. 2(a–t): Light microscopy (LM). *Chamaepinnularia thermophila* from the San Giovanni Su Anzu spring. Scale bar = 5 µm. **Fig. 2(u–v).** Scanning electron microscopy (SEM). *Chamaepinnularia thermophila* from the San Giovanni Su Anzu spring. **Fig. 2(u–v): external views.** Valves showing the straight raphe, with distal ends bent towards the same side, the central silica nodule and striae slightly radiate. **Fig. 2v: internal view.** Valve showing three large areolae interrupted by the silica deposition along the apical plane.

Comparison with Navicula tongatensis *Hustedt 1962*

The LM and SEM observations of *Chamaepinnularia thermophila* from Sardinia in Fig. 2(a–v) revealed identical valve outlines, structure of the raphe and striae pattern with *Navicula tongatensis* Hustedt reported in Fig. 3(a–u). Our specimens of *C. thermophila* show slightly longer and wider valves compared to the original description of Hustedt.

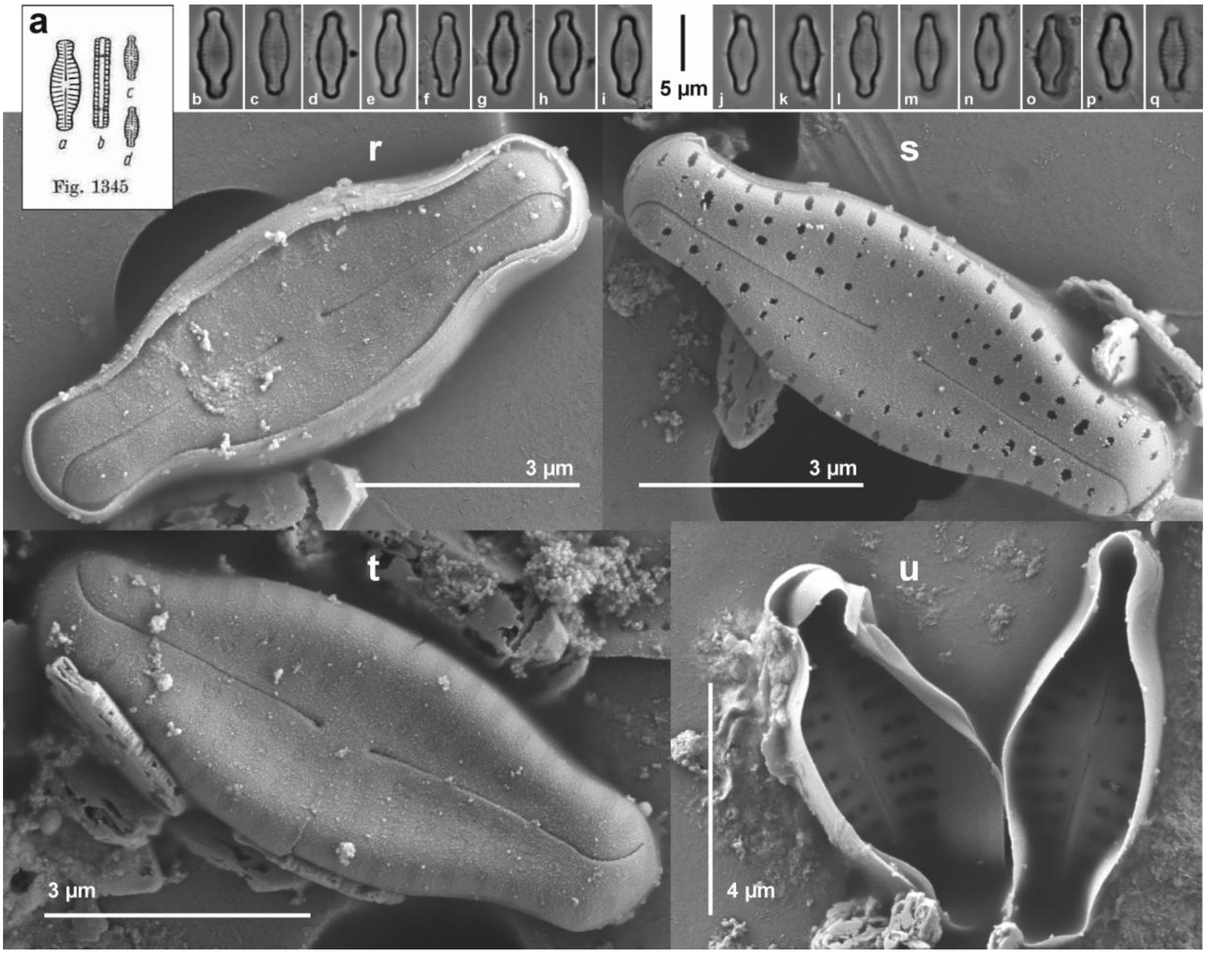

Fig. 3(a–u). Fig. 3a: Reproduction of the holotype material illustrated in Hustedt (1962) - Fig. 1345: a, b 2000/1, c, d 1000/1. **Fig. 3(b–q):** Light microscopy (LM). *Navicula tongatensis* Hustedt 1962 from his original material. Scale bar = 5 µm. **Fig. 3(r–u):** Scanning electron microscopy (SEM). *Navicula tongatensis* Hustedt 1962 from his original material. **Fig. 3r: external view.** Frustule showing unperforated girdle bands. **Fig. 3(s–t): external views.** Valves showing the radiate striae composed by three or four areolae and the straight raphe, with distal ends bent towards the same side. **Fig. 3u: internal views.** Valves showing the areolae interrupted by the silica deposition along the apical plane.

New finding in Sardinia (Italy)

Chamaepinnularia thermophila was found in summer 2016 (July) and winter 2017 (February) in samples collected from three substrates (cobbles, macrophytes and fine sediments) in the San Giovanni Su Anzu spring. Overall, the RA ranged from 0.9 to 10.8% and was higher in the cobbles (10.8%) and sediments (4.1%) in summer. The seasonal and spatial distribution of the species (winter/summer, substrates) is reported in Fig. 4.

The species composition based on the five most abundant taxa associated with *C. thermophila* for each substrate and season is presented in Fig. 5. Among these, the presence of *Cocconeis feuerbornii* Husted is interesting because it is considered a pantropical species (Pringle et al. 2016).

The spring was characterized by a constant temperature with a seasonal variation of 0.7 °C. Discharge and water depth were low. The conductivity was medium-high and consistent with the prevalent carbonate bedrock. Overall, the nutrient content, in particular the inorganic nitrogen compounds, was low. However, considering the phosphorus and BOD_5 values, the spring San Giovanni Su Anzu seems to be influenced by agricultural activities and livestock breeding present in the surrounding area. The results of physical and chemical variables of the site are reported in Table 2.

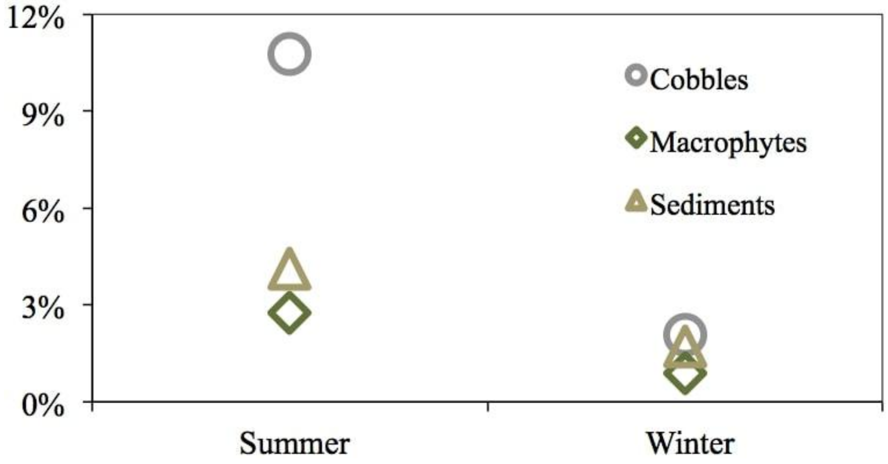

Fig. 4. Seasonal abundance of *Chamaepinnularia thermophila* in the three investigated substrates from the San Giovanni Su Anzu spring (Sardinia, Italy).

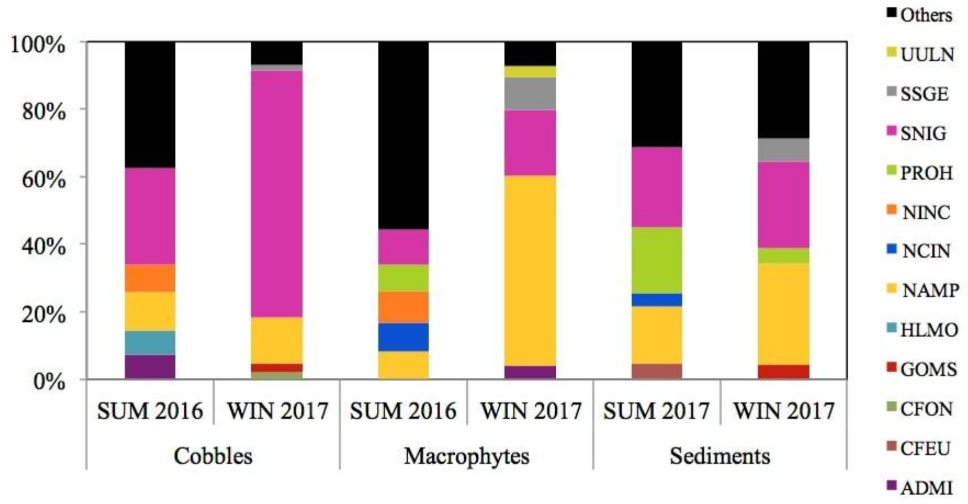

Fig. 5. Species composition based on the five most abundant taxa associated with *Chamepinnularia thermophila* for each substrate and season. Acronyms of the species: ADMI = *Achnanthidium minutissimum* (Kützing) Czarnecki; CFEU = *Cocconeis feuerbornii* Hustedt; CFON = *Caloneis fontinalis* (Grunow) Cleve-Euler; GOMS = *Gomphonema* sp.; HLMO = *Halamphora montana* (Krasske) Levkov; NAMP = *Nitzschia amphibia* Grunow; NCIN = *Navicula cincta* (Ehrenberg) Ralfs; NINC = *Nitzschia inconspicua* Grunow; PROH = *Planothidium rostratoholarcticum* Lange-Bertalot & Bąk; SNIG = *Sellaphora nigri* (De Notaris) C.E.Wetzel & Ector; SSGE = *Sellaphora saugerresii* (Desmazières) C.E.Wetzel & D.G. Mann; UULN = *Ulnaria ulna* (Nitzsch) Compère.

Table 2. Physical and chemical variables of the sites with *Chamaepinnularia thermophila* (including the new site of San Giovanni Su Anzu spring in Sardinia, Italy), relative abundance of the species and occurrence in different substrates.
** D.L. = Detection Limit. Abbreviations: cob = cobbles; mac = macrophytes; sed = sediments. Source of information and data of the spring sites in the French Antilles (Guadeloupe and Martinique): Brombach et al. 2000; ASCONIT Consultants 2015; Eulin-Garrigue et al. 2017.

	Guadeloupe		Martinique	Sardinia
Variables/Spring sites	Chute du Carbet	La Lise	Sucrerie	San Giovanni Su Anzu
Temperature (°C)	44.9	44.9	26.4	29.3–30
Flow rate (l s^{-1})				0.489–0.651
Mean water depth (cm)				6–7.3
pH (units)	6.51	7.33	7.92	7.26–7.30
Alkalinity (meq l^{-1})				3.8–4.7
Conductivity (µS cm^{-1})			1558	570–590
Dissolved Oxygen (mg l^{-1})			7.07	7.2–8.7
Oxygen (% saturation)			89.2	95–114
BOD_5 (mg O_2 l^{-1})				1.0–3.3
Cl^- (mg l^{-1})	368	27.5		42.5–46.1
Total hardness (mg $CaCO_3$ l^{-1})				235–235
Reactive phosphorus (µg P l^{-1})				16–24
Total phosphorus (µg P l^{-1})				37–78
N-NO_2^- (µg N l^{-1})				4–6
N-NO_3^- (µg N l^{-1})	200	1140		2448–2932
N-NH_4^+ (µg N l^{-1})				3–8
Total nitrogen (µg N l^{-1})				3743–6244
Reactive silica (mg Si l^{-1})	110	72		7.53–8.65
Total Suspended Solids (mg l^{-1})				0–1.8
Br- (mg l^{-1})				<D.L.–0.4
Ca^{2+} (mg l^{-1})	188	13.4		44–44
F^- (mg l^{-1})				<D.L.–0.1
Fe^{2+} (mg l^{-1})				0.04–0.04
HCO_3^- (mg l^{-1})	125	132		254.4–274.5
K^+ (mg l^{-1})	24	3.89		1.7–2.2
Mg^{2+} (mg l^{-1})	64.9	4.84		30.4–30.9
Mn^{2+} (mg l^{-1})	n.d.	0.011		0.04–0.05
Na^+ (mg l^{-1})	116.2	41.9		26–27.3
SO_4^{2-} (mg l^{-1})	303	5.69		8.0–9.8
% *Chamaepinnularia thermophila*	10-50%	<10%	55	0.9–10.8
Substrate			cob	cob/mac/sed

Geographic distribution

The type locality of the species (*Navicula thermophila*) is a spring from the Bouillante geothermal area in the Basse-Terre Island, in Guadalupe, French Antilles (Bourrelly & Manguin 1952). After its description, the species was reported in the following sites: 1) Spring of Chutes du Carbet in the Basse-Terre Island, Guadalupe, French Antilles; 2) Spring La Lise in the Basse-Terre Island, Guadalupe, French Antilles; 3) Spring Sucrerie in the Anses-d'Arlet sector, Martinique, French Antilles (ASCONIT Consultants 2015; Eulin-Garrigue et al. 2017).

The type locality of the species (*Navicula tongatensis*) is a well located near to Nuku'alofa in the Tonga Islands (Hustedt 1962), in the Polynesian archipelago, southwestern Pacific Ocean Hustedt (1962). After its description, the species was reported in three thermal springs in the southern border of the Kaokoveld region in Namibia, southwest Africa (Cholnoky 1966) and in freshwater diatom assemblages from Quaternary sections collected in the Pay-Khoy region (Moreyu River basin) in Russia (Loseva 1997).

According to Lange-Bertalot & Metzeltin (1996), the species (*Chamaepinnularia tongatensis*) was recorded in Borneo, in the Malay Archipelago, Southeast Asia. These authors also indicated a conspecificity of the *C. tongatensis* population from Borneo with *N. tongatensis* Hustedt. Finally, the species was reported in some ephemeral headwater streams in Czech Republic: 1) Suchá Bělá; 2) Písečná rokle; 3) Hluboký důl; 4) Mlýnská rokle; 5) Červený potok; 6) Vlčí potok by Veselá (2009) & Veselá & Johansen (2009). However, as pointed out by Veselá & Johansen (2009), the co-occurrence of the very similar *C. soehrensis* var. *capitata* (Krasske) Lange-Bertalot & Krammer in Lange-Bertalot & Metzeltin in these sites, suggests that the specimens observed may belong to a cryptic species.

A map of all sites with ascertained presence of *C. thermophila* (considering all its synonyms) is reported in Fig. 6. The most complete data sets for the sites with *C. thermophila* available in literature are reported in Table 2.

Fig. 6. Map of the sites of occurrence of *Chamaepinnularia thermophila* in the world. Brown circles: Quaternary sections; orange circles: thermal waters (springs and well); uncolored circles: unknown type.

Ecological traits of the species

According to available literature and our data, *Chamaepinnularia thermophila* was found mainly in thermal springs (20–60 °C), with alkaline pH (7.26–8), medium-high conductivity (570–1558 µS cm^{-1}) and low content of nitrates (200–2932 µg L^{-1}) (Bourrelly & Manguin 1952; ASCONIT Consultants 2015; Eulin-Garrigue et al. 2017). The species was very common in a well in the Tonga Islands with water temperature of 36 °C and pH 8.0 (Hustedt 1962). Overall, it was abundant in two thermal springs in South Africa (RA: 84% and 20.3%) (Cholnoky 1966), in the springs of French Antilles (RA: <10%–55%) (ASCONIT Consultants 2015; Eulin-Garrigue et al. 2017) and Sardinia (RA: 10.8%).

The presence of the species in Quaternary sections in Russia, suggest warmer water conditions in the past according to Loseva (1997).

In some ephemeral headwaters of Czech Republic, characterized by low temperatures (5.1–9.5 °C), pH (3.3–7.35) and conductivity (53–181 µS cm^{-1}), presumed specimens of the species were mostly rare (only one or two valves in each sample) (Veselá 2009; Veselá & Johansen 2009).

Discussion

The identity of *Chamaepinnularia thermophila* was clarified after structural analyses in LM and SEM on the original material of *Navicula thermophila* Manguin (Bourrelly & Manguin 1952) by Wetzel & Ector (2016).

Chamaepinnularia thermophila observed in Sardinia, showed morphological features such as shape of the valves, structure of the raphe and striae pattern corresponding with those reported by Wetzel & Ector (2016). Our specimens are slightly longer than those given by Manguin (Bourrelly & Manguin 1952). The number of striae is well correspondent with the specimens from the original material illustrated by Wetzel & Ector (2016), but slightly lower compared to the original description of the species.

Chamaepinnularia thermophila from Sardinia also showed a very high morphological similarity with *Navicula tongatensis* from Hustedt's original material. The valves show identical shape and terminal raphe deflections in the same direction. In both cases the striae are radiate and more spaced in the middle part of the valve and they are composed of three to four areolae externally occluded by hymens. The number of striae is also perfectly correspondent. The valves found in Sardinia are slightly longer and wider than those given by Hustedt (1962). Based on our observations, *C. thermophila* and *N. tongatensis* can be considered as synonyms of the same species.

Chamaepinnularia thermophila is a poorly known and probably rare species. In fact, in some cases, the specific location of the occurrence sites and their ecological characteristics are unclear or not available in literature and even considering all the synonyms of the species, the number of known sites remains limited. Furthermore, our extensive literature search revealed that *C. thermophila* is relatively abundant only in very few sites in the world, mainly in tropical areas. Limited number of sites and low abundance are often characteristics of rare diatoms (e.g. Noga & Rybak 2017).

Most of the known sites with *C. thermophila,* including the new site in Sardinia, are springs characterized by warm waters, alkaline pH and medium-high/high conductivity.

These characteristics seem to confirm the ecological preferences initially hypothesized by Manguin: "halophilous species of salt thermal springs" (Bourrelly & Manguin 1952). For the trophic level, a comparison among sites was possible only for nitrates with La Lise and Chute du Carbet springs (Brombach et al. 2000), suggesting a possible preference of the species for low concentrations.

However, our values of phosphorus and BOD_5 do not reflect the condition of pristine environments, suggesting a possible tolerance of the species for moderate contents of nutrients and organic matter. Our data provide first information on the distribution of *C. thermophila* at small seasonal and spatial scale (winter/summer-substrates). Overall, cobbles, characterized by highest relative abundance of the species in both seasons (summer 2016 and winter 2017), seems to be most favorable substrate than macrophytes and sediments. Further, in summer, the species was more abundant in all investigated substrates, probably favored by a slightly higher water temperature and wider light availability. Our data seem to confirm the preference of the species for warmer waters. The occurrence of *C. thermophila* in ephemeral headwaters of Czech Republic (Veselá 2009; Veselá & Johansen 2009) with very different environmental characteristics compared to the spring sites, seem unusual and should be better investigated. However, the uncommon and rare presence of the species reported in these environments, could suggest a possible tolerance for colder waters, acid to slightly alkaline pH and low conductivity and especially for seasonal desiccation episodes. This ability to withstand the dry period could be an important biological trait, considering that several *Chamaepinnularia* species are characteristic of aerial habitats (Wetzel et al. 2013).

This study highlights the synonymy of *C. thermophila* and *N. tongatensis*, considered as two different species for a long time after their description, contributing to uniform the nomenclature used so far. It also provides a first framework on the global geographic distribution and ecology of the species based on the information available in literature and our new data. The occurrence of this species as well as other rare diatoms in springs located in very distant and different geographic areas, especially islands, is a deserving aspect of further future studies. In fact, springs are understudied habitats (Mogna et al. 2015).

In addition, they are considered in themselves as water islands due to their scattered distribution (Werum 2001; Cantonati et al. 2012) and the mechanisms of dispersion for diatoms are passive and limited (Vanormelingen et al. 2008).

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8. Chapter IV

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Sellaphora gologonica sp. nov. (Bacillariophyta, Sellaphoraceae), a new diatom species from a Mediterranean karst spring (Sardinia, Italy)

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Abstract

A new epilithic *Sellaphora* (Bacillariophyceae) species from a karst spring of Sardinia is described based on light and scanning electron microscopic observations. *Sellaphora gologonica sp. nov.* shares similarities with small-celled *Sellaphora* previously described from Europe and particularly with *S. atomoides*, *S. hafnearae* and *S. seminulum*. *Sellaphora gologonica* seems to be related to *S. seminulum* based on the striae patterns and valve ultrastructure, as well as the sinuous raphe system. The main differences between *S. gologonica* and *S. seminulum* lies in its valve shape (small, elliptic to oval), not presenting capitated nor protracted apices, as in the later taxon. *Sellaphora gologonica* was discovered in freshwater habitats with slightly alkaline and oligotrophic waters presenting medium hardness and mineralization, at the smaller water emergence of karst spring system Su Gologone (Sardinia).

Keywords: epilithic diatoms, karst waters, *Sellaphora* species, scanning electron microscopy, spring biodiversity, taxonomy

Introduction

The genus *Sellaphora* defined by Mereschkowsky (1902: 186) and updated by Mann (1989: 2), presents valve outlines that range from linear-lanceolate to elliptical, with bluntly rounded or capitate apices (Mann *et al.* 2008). The structure of the frustule in the genus *Sellaphora* has been described in detail in a comparative account of *Sellaphora* and *Navicula* by Mann (1989), including some aspects previously described by Kaczmarska (1979), Schoeman & Archibald (1979) and Sims & Paddock (1979). The striae are usually fine and uniseriate. They are often slightly radiate and more widely spaced in the central part of the valve, and usually parallel or convergent towards the poles (Mann 1989). The areolae are small and round, occluded by a hymen (Mann 1981); they not form longitudinal striae and are difficult to distinguish in light microscope. The raphe has external fissures straight or sinuous, slightly deflected at the centre, where they end in expanded pores, and curved at both poles. Internally, the raphe endings are deflected at the centre. The distal endings form a long narrow helictoglossa (Mann 1989).

The genus *Sellaphora* plays a key role in the understanding of species limits in diatoms by using traditional morphological criteria (Potapova & Ponader 2008). In fact, long-term studies, based also on genetic analyses, revealed that slight morphological differences could indicate the presence of several species within previously broadly defined morphospecies (Mann *et al.* 2004, 2008). Recent studies on several original materials have transferred some naviculoid species from *Navicula* Bory (1822: 128) in the broad sense, *Naviculadicta* Lange-Bertalot in Lange-Bertalot & Moser (1994: 83) and *Eolimna* Lange-Bertalot & W. Schiller in Schiller & Lange-Bertalot (1997: 166) to the genus *Sellaphora*, on the basis of the morphology and geometric morphometric analyses (Falasco *et al.* 2009, Wetzel *et al.* 2015). Currently, *Sellaphora* is a large genus that includes several small-celled species (Mann *et al.* 2008, Wetzel *et al.* 2015, Beauger *et al.* 2016). According to the last update, it holds 159 species of which 62 uncertain and 1 nomen nudum, according to DiatomBase (Kociolek *et al.* 2017).

Springs are highly diverse environments with a wide range of physical and chemical conditions and variety of microhabitats that contribute significantly to local and regional biodiversity (Sabater & Roca 1992, Angeli *et al.* 2010,

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Cantonati *et al.* 2012, Wojtal 2013). They are often rich of diatom species and can host a larger number of Red List taxa than other aquatic habitats (Cantonati *et al.* 2012). Despite their importance for biodiversity and water quality, springs are largely disregarded by freshwater monitoring and are not explicitly considered by the EU Water Framework Directive (Segadelli *et al.* 2015).

Species of the genus *Sellaphora* are widespread in alkaline freshwaters from temperate and tropical areas where they are often among the most abundant benthic diatoms, but also in brackish waters (Wetzel *et al.* 2015, Beauger *et al.* 2016). Recently, a new *Sellaphora* called *S. perhibita* (Hustedt) Lange-Bertalot & Cantonati in Cantonati & Lange-Bertalot (2010) was found in springs on carbonate substratum of the Berchtesgaden National Park (north-eastern Alps, Germany). In 2014, during a survey in the French Massif Central, a new *Sellaphora* called *S. labernardierei* Beauger, C.E. Wetzel & Ector in Beauger *et al.* (2016) was found in a mineral spring of France.

Different studies on diatoms from springs were done all over the world and some new diatom taxa were described (e.g. Sabater & Roca 1992, Werum & Lange-Bertalot 2004, Potapova & Ponader 2008, Wojtal 2009, 2013, Angeli *et al.* 2010, Cantonati & Lange-Bertalot 2011, Kapetanović *et al.* 2011, Żelazna-Wieczorek 2011, Solak & Wojtal 2012, Isheva & Ivanov 2016). These studies allowed a better knowledge of the different taxa occurring in the springs and their ecology (Wojtal 2009, Stavreva-Veselinovska & Todorovska 2010). Moreover, the presence of these new species underlines the importance of protecting these unique habitats that offer a reservoir of threatened or declining species. The present paper describes a new species of the genus *Sellaphora* from a karst spring of Sardinia, the second largest island of the Mediterranean basin, and documents its morphology with light microscopy (LM) and scanning electron microscopy (SEM). A morphological comparison is made with the most similar *Sellaphora* taxa and its ecology is also addressed.

Material and methods

Description of study site

Sa Vena is the smaller spring of the Su Gologone karst system, located at the foot of the Supramonte massif, in the central-eastern Sardinia (Fig. 1). The Supramonte massif is characterized by impervious and largely inaccessible mountains, high level of naturalness and scarce human presence, with no stable settlements (De Waele 2008). The hydrogeological basin that feeds the spring covers a total surface area of about 160 km^2 and is composed of Middle Jurassic-Upper Cretaceous dolostones and limestones covering a crystalline Palaeozoic basement made out of granites and metamorphic rocks (Cabras *et al.* 2008).

The Su Gologone spring system, the most important at regional level for its discharge (mean minimum of approximately 100 L s^{-1} and maxima of approximately 10,000 L s^{-1} during storm events) (De Waele 2008). It was recognized as Natural Monument of Sardinia (R.D. 845/1998) and as a Zone of Respect (L.D. 152/1999). It is inside the Gennargentu and the Gulf of Orosei National Park (D.P.R. 30 March 1998), the Site of Community Importance and the Zone of Special Protection "Supramonte di Oliena, Orgosolo e Urzulei-Su Sercone" (ITB022212) for the biogeographic Mediterranean region.

Sa Vena (Fig. 2) is a perennial rheocrenic spring that flows at 103.7 m a.s.l. from a small fracture of the rock connected to the main karst network (Sanna *et al.* 2002). The potential disturbances for the spring system are the sheep breeding, although less important than in the past, the intensive animal farming in the allochthonous recharge area, in the southern part of the aquifer, and the tourism (De Waele 2008). Part of the water is captured for local drinking purposes with average withdrawals of about 100 L s^{-1} (Bianco 1993). In addition, the spring is subject to flood events due to the overflow of the Pedra′e Othoni dam on the Cedrino River after intense rainfalls (Bianco 1993, De Waele 2008).

Diatom sampling and environmental analyses

Epilithic diatoms were collected on a complete seasonal cycle during 2014 (winter: January-February 2014; spring: April 2014; summer: July 2014; autumn: October 2014), with two replicates collected in the following winter (January 2015) and spring (April 2015) seasons. Sampling was carried out by scraping the upper surface of hard natural substrates (five cobbles randomly selected in flowing water, for a total surface area of at least 100 cm^2) with a hard-bristled toothbrush, following the methods reported in Kelly *et al.* (1998) and ISPRA (2014). All diatom samples were preserved in 100 mL polyethylene bottles and fixed *in situ* with a formaldehyde solution (to a final concentration of 4% v/v).

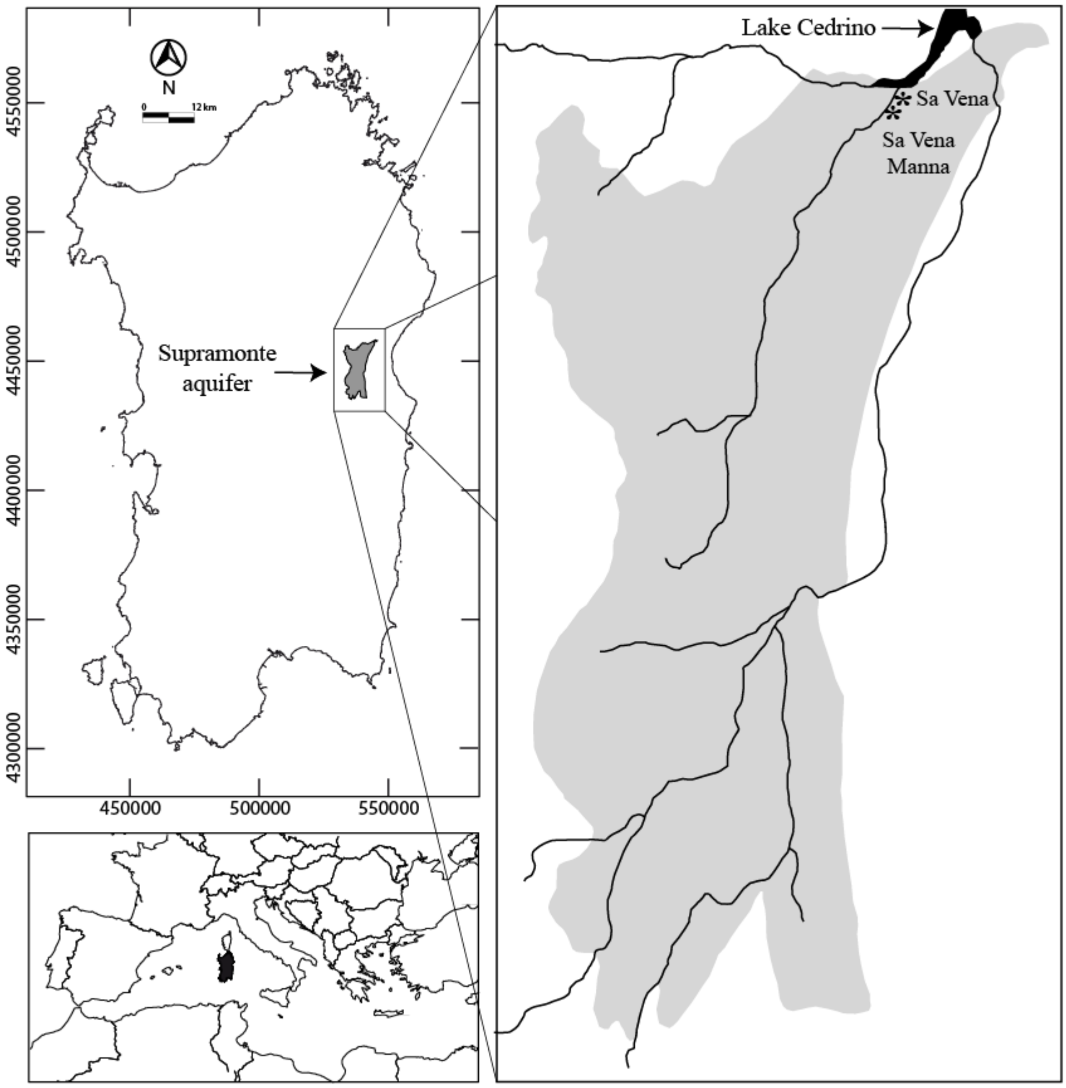


FIGURE 1. Geographic location of the Sa Vena spring (Su Gologone karst system).

Water samples were collected monthly, from January 2014 to April 2015, using 1 L polyethylene bottles and were preserved in cold and dark conditions for the physical and chemical analyses in the laboratory. Temperature, pH, conductivity and percentage of oxygen saturation were measured *in situ* with a multiparameter probe (YSI ProPlus). Alkalinity, dissolved oxygen, chlorides (Cl^-), total hardness, biological oxygen demand (BOD_5), total suspended solids (TSS) and some ions (Ca^{2+}, Mg^{2+}, Fe^{2+} and Mn^{2+}) were measured in the laboratory using standard methods reported by APHA (1998) and APAT/IRSA–CNR (2003). Soluble reactive phosphorus ($P\text{-}PO_4^{3-}$), total phosphorus (TP), ammonium ($N\text{-}NH_4^+$), nitrites ($N\text{-}NO_2^-$), nitrates ($N\text{-}NO_3^-$), total nitrogen (TN) and reactive silica (RSi) were analysed using methods reported in Strickland & Parsons (1972).

Diatom analyses

Small sub-samples of raw material were prepared for LM observation following the method described in Prygiel & Coste (2000). Samples were cleaned using hydrogen peroxide (H_2O_2, 35% v/v) and hydrochloric acid (HCl, 37% v/v), and rinsed several times. Cleaned material was diluted with distilled water to avoid excessive concentrations of diatom valves on the slides and mounted in Naphrax®. LM observations were done using a Leica® DM2700M microscope with 100x oil immersion objective using a differential interference contrast. Morphometric measurements were performed using a Leica® DMRX bright field microscope with 100x oil immersion objective, and light photographs were taken with a Leica® DC500 camera.

FIGURE 2. Sa Vena spring: water emergency point **(a)**, brook **(b)**, water abstraction system inside the rock **(c)**.

For the scanning electron microscopy (SEM), parts of the oxidized suspensions were filtered with additional deionized water through a 3 µm Isopore™ polycarbonate membrane filter (Merck Millipore). Filters were mounted on aluminium stubs and coated with platinum using a Modular High Vacuum Coating System BAL-TEC MED 020 (BAL-TEC AG, Balzers, Liechtenstein). An ultrahigh-resolution analytical field emission (FE) scanning electron microscope Hitachi SU-70 (Hitachi High-Technologies Corporation, Japan) operated at 5 kV and 10 mm distance was used for the analysis. SEM images were taken using the lower (SE-L) and upper (SE-U) detector signal and sometimes tilted up to an angle of 28 degrees. Photographs were digitally manipulated and plates containing light and scanning electron microscopy images were created using CorelDraw X6. Samples and slides are stored at the University of Sassari (Department of Architecture, Design and Urban Planning) and the Botanic Garden Meise, Belgium (BR).

Sellaphora seminulum was considered by us as the most similar species, thus additional SEM from Grunow's original material [*Navicula seminulum* Grunow 1860 from "Radegund", Liechtenstein housed in the *Naturhistorisches Museum* (W), Vienna (Grunow collection, n° 1580, acquisition 1901)] was used for comparison and illustration.

Morphological terminology follows Ross *et al.* (1979) and Round *et al.* (1990) for terminology applied to striae and areolae, Barber & Haworth (1981) for terminology related to valve shape and striae orientation.

Results

Physical and chemical characteristics of the spring

The mean water temperature of the spring was 12.9 °C from January 2014 to April 2015. The values showed low oscillations over time, ranging from 11.5 °C in January to 14.5 °C in May (unusually hot month). The pH was slightly alkaline (7.3–8.3) and the water oxygenation level was good, with percent saturation generally >75%. The hardness (135–175 mg $CaCO_3$ L^{-1}) and mineralization level (241–379 µS cm^{-1}) were medium. The content of Cl^- was relatively low (10.6–24.8 mg L^{-1}). Ca^{2+} (27.5–54 mg L^{-1}) was more abundant than Mg^{2+} (6.1–21.9 mg L^{-1}) in all months. The BOD_5 values (0.8–7.4 mg L^{-1}) revealed the presence of a moderate organic contamination, with peaks during some months. Nutrients showed wide ranges of variation but were relatively low. The P-PO_4^{3-} annual mean was 8.5 µg L^{-1} with values ranging between 5 (November) and 13 (August) µg P L^{-1}. TP, with an annual mean value of 22 µg L^{-1}, was highest in January and February 2014 (>60 µg P L^{-1}). N-NO_3^- was the most abundant inorganic nitrogen compound, with an annual mean value of 614.3 µg N L^{-1}, providing the greatest contribution to TN, which ranged from 493 (May) to 2,490 µg N L^{-1} (August). TSS values were always very low, showing a maximum of 2.9 mg L^{-1} in May. The results are summarised in Table 1.

Species description

Division **Bacillariophyta**
Class **Bacillariophyceae**
Subclass **Bacillariophycidae**
Order **Naviculales**
Suborder **Sellaphorineae**
Family **Sellaphoraceae**
Genus ***Sellaphora***

Sellaphora gologonica G.G. Lai, Ector & C.E. Wetzel *sp. nov*. (Figs 3–55)

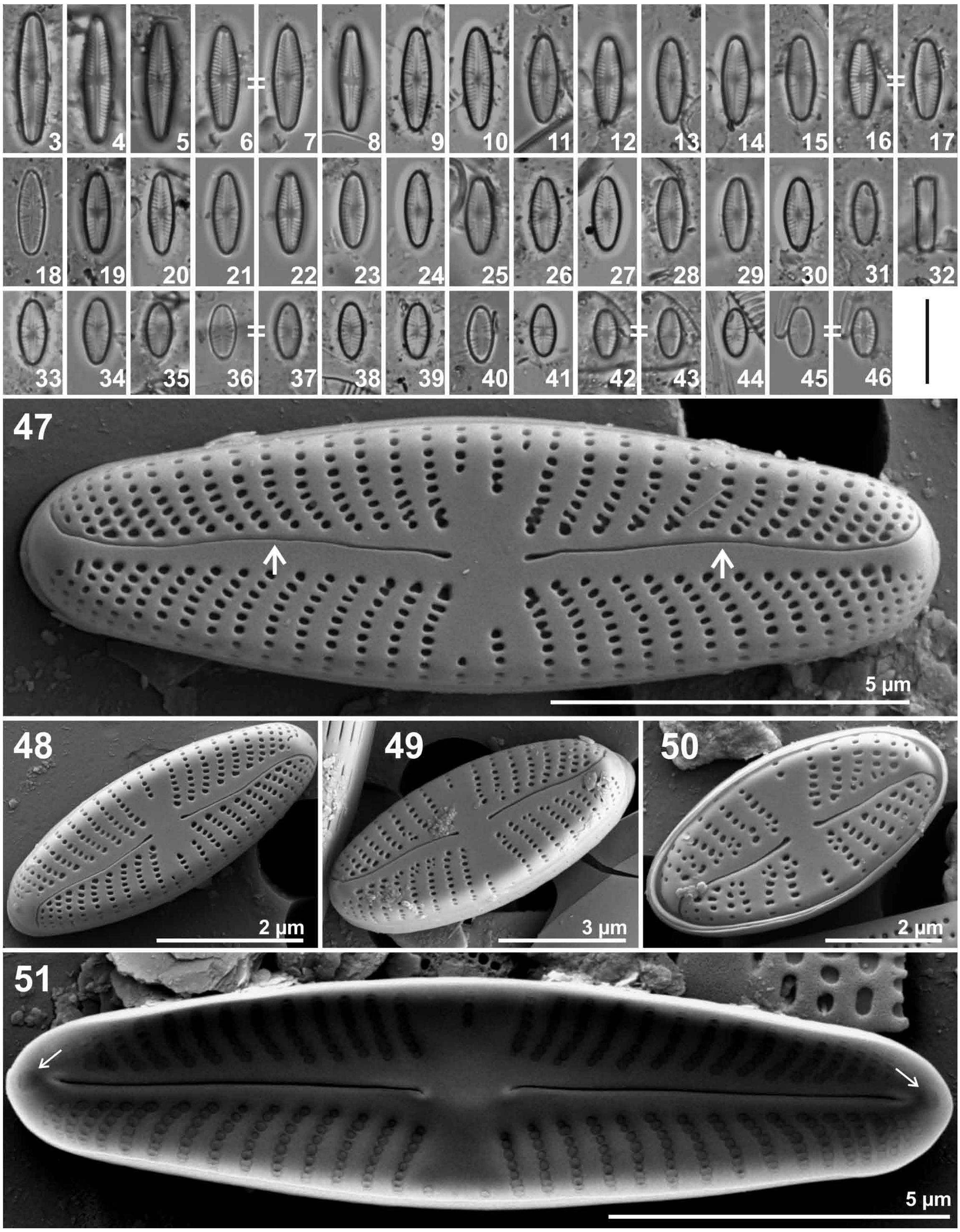
Description:—Light microscopy observations (Figs 3–46): Valves are linear-elliptic to linear-lanceolate with rounded ends. Valve dimensions (n=45): length 8.3–13.3 µm, width 3.4–4.2 µm. Central area is large, bow-tie shaped, bordered by irregularly shortened striae. The striae are slightly radial and about 20–24 in 10 µm. The axial area is slightly narrow and linear over most of the valve. The raphe slits are sinuous with enlarged proximal raphe endings. — Scanning electron microscope observations (Figs 47–55): Externally, the raphe is sinuous and the drop-like proximal raphe endings are slightly deflected to the same side (Fig. 47). Distal raphe fissures are strongly bent to the same side of the valve, and extended onto the mantle (Figs 49–51). The striae are uniseriate, sometimes with double areolae near the axial area (Figs 49–50). At the central area three shorter striae can be present (Figs 49–51). Internally, the proximal raphe endings are slightly deflected and the distal raphe fissure ends in an inconspicuous helictoglossa (Fig. 46). Each areola is internally occluded by a hymen (Fig. 47). A large rounded apical pit is present at both apices (Fig. 51); this is usually closed externally.

Type:—ITALY. Nuoro: Su Gologone (Oliena), 103.7 m a.s.l., 40°17.361' N, 009°29.772' E, collection date 21 January 2014 (designated here, holotype: slide no. BR-4504 Botanic Garden Meise, Belgium. Specimen here depicted in Figs 6–7).

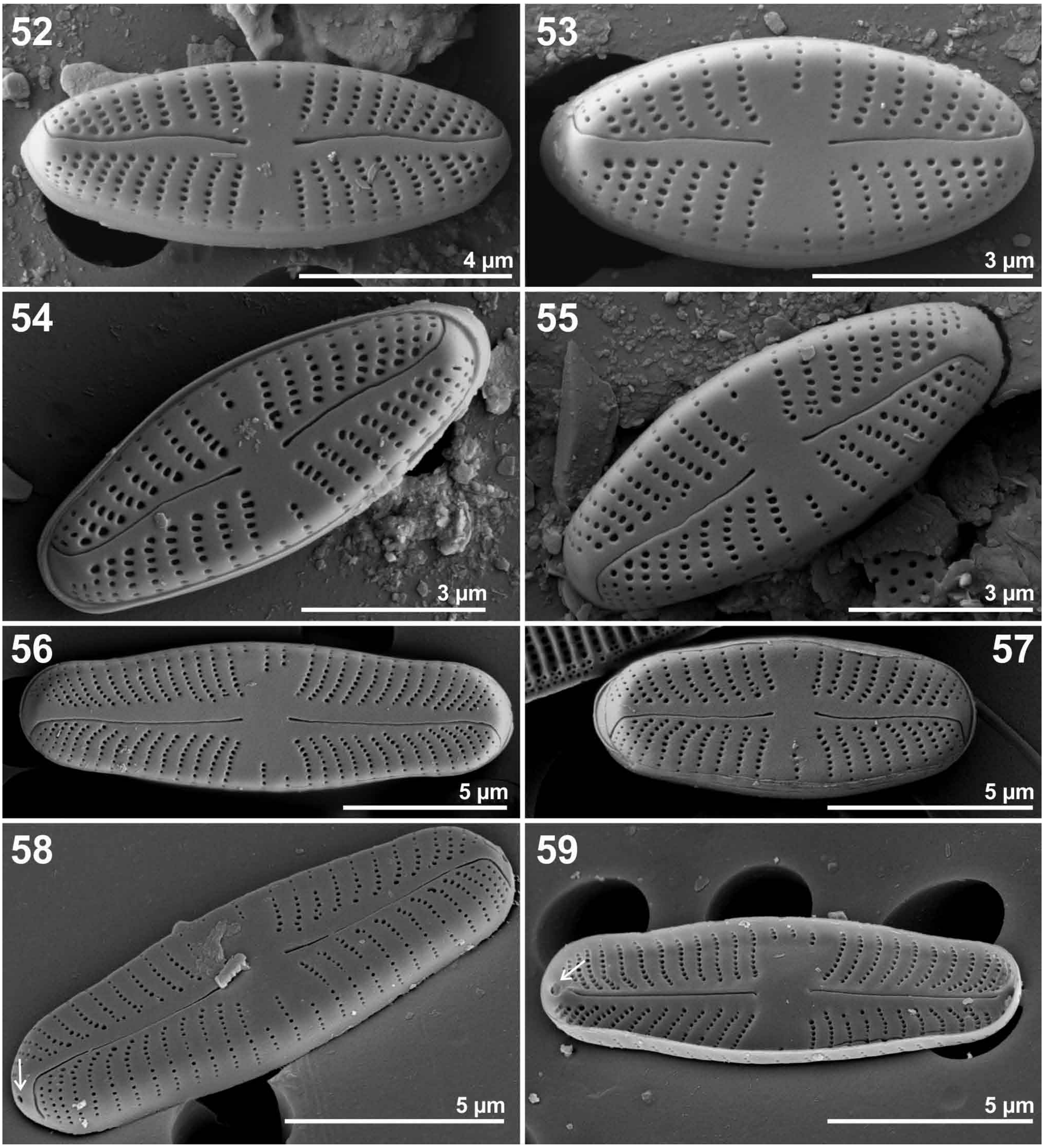
Etymology:—The name of the new species *S. gologonica* refers to the name of the spring system "Su Gologone" from the local hydronym Gol(o)gone = big whirlpool or siphon.

TABLE 1. Minimum, maximum, mean and standard deviation of the physical and chemical parameters recorded in the field and analysed in the laboratory (n = 15 measures) (D.L.: Detection Level).

Variables	min	max	mean±stand dev
Temperature (°C)	11.5	14.5	12.9±1.2
pH (units)	7.3	8.3	7.7±0.5
Conductivity (µS cm^{-1})	241	379	331±46.0
Alkalinity (meq L^{-1})	2.4	3.3	2.9±0.1
Dissoved Oxygen (mg L^{-1})	6.5	10.9	9.3±0.5
Oxygen saturation (%)	63	104	87±7.8
BOD$_5$ (mg L^{-1})	0.8	7.4	3.5±2.5
Cl$^-$ (mg L^{-1})	10.6	24.8	18.5±3.8
Total hardness (mg CaCO$_3$ L^{-1})	135	175	158±24.7
P-PO$_4^{3-}$ (µg P L^{-1})	5	13	8.5±2.1
TP (µg P L^{-1})	7	64	22±38.9
N-NH$_4^+$ (µg N L^{-1})	<D.L.	36	18.5±21.9
N-NO$_2^-$ (µg N L^{-1})	<D.L.	311	2.5±0.7
N-NO$_3^-$ (µg N L^{-1})	<D.L.	952	614.3±17.7
TN µg (N L^{-1})	493	2490	1204.1±169.7
RSi (mg Si L^{-1})	1.6	2.1	1.9±0
Ca^{2+} (mg L^{-1})	27.5	54	44.7±4.2
Mg^{2+} (mg L^{-1})	6.1	21.9	12±8.6
TSS (mg L^{-1})	0.2	2.9	0.8±0.2



FIGURES 3–51. *Sellaphora gologonica* G.G. Lai, Ector & C.E. Wetzel *sp. nov.* Population "Su Gologone" spring. LM (Figs 3–46): size reduction series of the population. Specimen here depicted in Figs 6–7. Frustules rectangular in girdle view (Fig. 31). SEM (Figs 47–51): Figs 47–50. External views showing striae patterns (strongly radiate) and double areolae near the axial area on the valve surface. Arrows–sinuous raphe system. Fig. 51. Internal view showing slightly deflected raphe endings. Arrows–large rounded apical pits present at both apices. LM scale bar = 10 µm.



FIGURES 52–59. SEM comparison between *Sellaphora gologonica sp. nov.* from Su Gologone spring (Sardinia) and *Sellaphora seminulum* from Grunow's original material. Figs 52–55. External views of *S. gologonica sp. nov.* Figs 56–58. External views of *S. seminulum*. Fig. 59. Internal view of *S. seminulum*. Arrows–apical pits present at apices.

Associated diatom species

The type population of *S. gologonica sp. nov.* was found at Sa Vena spring associated with several other taxa. *Sellaphora gologonica* was rare in October 2014 with relative abundance <1.5% (0.2%) and frequent in January 2015 with relative abundance between 1.5 and 5% (2%). It was not present in the counts in all other months. In October 2014 the three dominant taxa in the diatom community were *Achnanthidium minutissimum* (Kützing 1833: 578) Czarnecki (1994: 157) (33.6%), *A. subatomus* (Hustedt 1939: 554) Lange-Bertalot (1999: 279) (15.7%) and *Amphora pediculus* (Kützing 1844: 80) Grunow in Schmidt *et al.* (1875) (12.7%). The frequent taxa (relative abundance between 1.5

and 5%) were respectively: *Planothidium lanceolatum* (Brébisson ex Kützing 1846: 247) Lange-Bertalot (1999: 287) (4.9%), *Cocconeis neothumensis* Krammer (1990: 151–152) (3.5%), *Achnanthidium lineare* W. Smith (1855: 8) (2.7%), *Meridion circulare* (Greville 1823: pl. 35) C. Agardh (1831: 40) (2.7%), *Amphora indistincta* Levkov (2009: 69–70, 287) (2%), *Nitzschia dissipata* (Kützing 1844: 64) Rabenhorst (1860: 948) (2%), *Cocconeis placentula* Ehrenberg (1838: 194) (1.9%), *Gomphonema elegantissimum* E. Reichardt & Lange-Bertalot in Hofmann *et al.* (2011: 302) (1.9%) and *Caloneis fontinalis* (Grunow in Van Heurck 1885: 103) Cleve-Euler (1932: 130) (1.7%). In January 2015 the three dominant taxa in diatom community were *A. minutissimum* (Kützing) Czarnecki (28%), *A. subatomus* (Hustedt) Lange-Bertalot (18.4%) and *Amphora pediculus* (Kützing) Grunow (10.1%). The frequent taxa (relative abundance between 1.5 and 5%) were respectively: *P. lanceolatum* (4.3%), *A. indistincta* Levkov (3.8%), *N. dissipata* (Kützing) Rabenhorst (3.8%), *C. fontinalis* (Grunow) Cleve-Euler (3.6%), *C. placentula* Ehrenberg (3.6%), *C. neothumensis* Krammer (3.1%), *C. pseudolineata* (Geitler 1927: 515) Lange-Bertalot in Werum & Lange-Bertalot (2004: 133) (2.7%) and *C. euglypta* Ehrenberg (1854: 8, pl. 34, part 6-A, fig. 2) (2.5%).

Discussion

The new species *Sellaphora gologonica* presents a set of morphological characters such as the valve dimensions, the valve outline, the structure of the raphe and the striation pattern, which clearly places it into the genus *Sellaphora*.

Sellaphora gologonica sp. nov. shows similarities with three small *Sellaphora* species such as *Sellaphora atomoides* (Grunow in Van Heurck 1880: (p. 107), pl. 14, fig. 12) C.E. Wetzel & Van de Vijver in Wetzel *et al.* (2015: 219–221), *S. hafnearae* Kapetanović & Jahn in Kapetanović *et al.* (2011: 132) and *S. seminulum* (Grunow 1860: p. 552, pl. 2, fig. 3a–d) D.G. Mann (1989: 2) (Table 2).

TABLE 2. Main characteristics of the *Sellaphora gologonica sp. nov.* and similar small species.

	Sellaphora gologonica sp. nov.	*Sellaphora atomoides*	*Sellaphora hafnearae*	*Sellaphora seminulum*
Valve length	8.3–13.3	3.4–16.3	9.9–16.7	9.8–16.5
Valve width	3.4–4.2	2.6–3.7	3.0–3.6	3.4–4.4
Striae in 10 µm	20–24	30–36	23–24	18–22
Valve shape	linear-elliptic to linear-lanceolate	linear-elliptic to linear-lanceolate	linear-elliptic	linear–lanceolate to lanceolate, inflated in the middle portion
Pole shape	rounded, non-protracted	rounded, non-protracted	capitate	protracted, broadly rounded, slightly rostrate
Central area shape	bow-tie shaped	bow-tie shaped	± rectangular	butterfly
Striae	uniseriate (rarely biseriate near the axial area)	uniseriate	uniseriate	uniseriate (rarely biseriate near the axial area)

The valve outline of *S. gologonica sp. nov.*, linear-elliptical with rounded apices, is similar to *S. atomoides*. However, *S. gologonica* has a sinuous raphe system while in *S. atomoides* the raphe is straight. The two species show an overlapping size range. *S. atomoides* has slightly narrow valves (width 2.6–3.7 µm) than *S. gologonica* (3.4–4.2 µm).The central areas have a similar shape. The striae are radiate in both taxa but they are always uniseriate in *S. atomoides* while can present double areolae near the axial area in *S. gologonica.* Moreover, *S. atomoides* shows higher striae density (30–36 striae in 10 µm) than *S. gologonica* (20–24 striae in 10 µm).

Sellaphora gologonica sp. nov. and *S. seminulum* have a distinct shape. *S. seminulum* is inflated in the middle (Figs 56–59). Also in this case the two species show an overlapping size range and axial and central areas with similar shape. The striae bordering the central area, which is wide, symmetrical, bow-tie shaped, and usually extends to close to the junction of valve face and mantle, are slightly but noticeably curved; two to three shorter striae, often uneven in length, are present in the very middle of the valve. Like *S. seminulum*, *S. gologonica* presents a sinuous raphe; and externally the drop-like proximal raphe endings are deflected to the same side. The variability of the striae pattern is similar in *S. gologonica* and *S. seminulum* (see Wetzel *et al.* 2015, fig. 298): both shows strongly radiate and uniseriate striae, sometimes partially biseriate near the axial area (mainly in smaller valves).

Sellaphora gologonica sp. nov. has a different valve outline in respect to *S. hafnearae.* The two taxa differs also in their size range. *Sellaphora hafnearae* appears longer (9.9–16.7 µm) and narrower (3.0–3.6 µm). The central area is almost rectangular in *S. hafnearae* and bow-tie-shaped in *S. gologonica.* The striae are radial in both taxa, more dense (23–24 in 10 µm) and with larger areolae in *S. hafnearae*. Although *S. hafnearae* resembles to *S. gologonica* in LM, the two taxa differs quite substantially in SEM. *Sellaphora hafnearae* presents strongly silicified structures with a strongly developed virgae internally, and conspicuous apical polar bars with a clear interruption of the areolae (both externally and internally) near the apices. The raphe slits are also distinct in the two taxa. *Sellaphora hafnearae* has indistinct central pores and a straight raphe.

The habitat and autecology of *S. atomoides* and *S. seminulum* are currently not precisely known due to confusion of these species with other small similar taxa (Lange-Bertalot *et al.* 2017). *Sellaphora hafnearae* was found so far only in the mineral rich part of the oligotrophic fen Bijambare in Bosnia & Herzegovina (Kapetanović *et al.* 2011). *Sellaphora gologonica sp. nov.* was found in fresh and slightly alkaline waters, with a medium hardness and mineralization, a low trophic level, comparable with that of the Alpine springs, and a moderate organic contamination. The presence of organic matter is attributable to pasture activities carried out in the surrounding area and the pollutant load of the Cedrino River, which submerges the spring after intense rainfalls. The co-occurrence of taxa with a wide ecological range such as *Amphora pediculus*, *Cocconeis placentula*, *C. pseudolineata* (xenosaprobous-/β-mesosaprobous-/polysaprobous) and *C. euglypta* and *Nitzschia dissipata* (oligosaprobous-/α-mesosaprobous) (Van Dam *et al.* 1994, Dell'Uomo 2004) among the abundant and frequent species, indicates the influence of organic matter on the diatom community.

Conclusions

Karst springs are strategic water sources and potential hotspots of biodiversity, especially in the Mediterranean area. In addition they are considered among the most vulnerable groundwater-dependent ecosystems (GDEs) for both water quantity and quality. In Sardinia, knowledge of features and hydrogeological behaviour of karst springs is fragmentary and their biocenoses and ecological dynamics are mostly unknown. This study describe a new species of the genus *Sellaphora* from Su Gologone spring system, the most important of the Island. It provides a morphological comparison with some small similar *Sellaphora* taxa as useful support for their taxonomic distinction and describes the main environmental characteristics of the spring habitat. The population of *S. gologonica sp. nov.* was associated with several other diatom taxa and the spring showed high diatom diversity, despite the presence of anthropogenic disturbance sources, such as the water abstraction. This study offers a preliminary contribution to knowledge on diatom flora and ecology of karst springs of Sardinia and more in general of the Mediterranean area, especially islands.

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9. Chapter V

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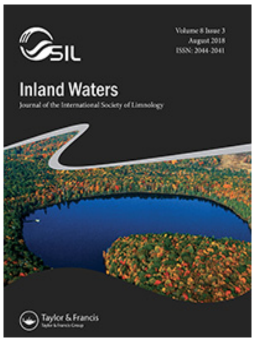

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ABSTRACT
In Sardinia, Italy, karst springs are strategic water resources and potential hotspots of biodiversity; however, they are understudied, and their communities remain largely unknown. This study was conducted at Sa Vena spring (Su Gologone system) to characterize the diatom communities, to describe their relationships with environmental variables, and to evaluate the impact of an extreme flash flood. High species richness, diversity, and evenness were found both in epiphytic and epilithic assemblages. Statistical analyses showed significant differences between assemblages from the 2 substrates and in assemblages from the same substrate. Differences seem due to the stronger impact of seasonal water level variations and flash flood on macrophytes than on cobbles. Further, epilithic assemblages showed more stable behavior over time, both on an annual (higher resistence) and a multiannual (higher resilience) scale. Alkalinity ($p = 0.002$) and dissolved oxygen ($p = 0.004$) explained a significant amount of variance in epiphytic and epilithic assemblages, respectively, according to RDA analyses; however BOD_5, negatively correlated with dissolved oxygen, can be the most probable driver of epilithic diatoms. The results suggest the importance of the proper management and preservation of this biotope, both for the high biodiversity and potential vulnerability to climate change.

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Introduction

Springs are among the most representative surface components of karst systems (Ford and Williams 2007). They contribute to the maintenance of numerous surface aquatic ecosystems and wetlands (Ravbar and Kovačič 2015) and host a wide variety of ecological niches (Culver and Pipan 2009) with rich and diversified biocenoses (Wehr and Sheath 2015). Karst springs also provide good quality waters (Stevanović 2015) that may require light treatments to achieve compliance with drinking water standards (e.g., Koch et al. 2013). According to UNESCO, they are the most significant and safest source of drinking water (Aureli 2010) and supply ∼25% of the world's population (Ford and Williams 2007).

The flow rate of karst spring is usually high and undergoes changes between the wet and dry seasons (Petrič and Rubinić 2015). Floods and flash floods may occur from individual storm events, inducing changes in the environmental conditions and biota (Dedić et al. 2015). The food web of springs depends on algae as the main energy source (Wehr and Sheath 2015). Among microalgae, diatoms are an abundant taxa-rich group and contribute to the biogeochemical processes. They are also useful indicators of many spring characteristics (Cantonati et al. 2012).

Because of their peculiar hydromorphological features, karst springs are particularly vulnerable to natural and anthropogenic stressors (Delle Rose et al. 2007). In Mediterranean countries they are strongly influenced by a highly irregular rainfall regime and threatened by climate change and water abstraction (e.g., IPCC 2007).

In Sardinia, Italy, the second largest island of the Mediterranean basin, karst areas cover 2088 km^2 (8.7% of the total surface). Springs are strategic water resources and potential hotspots of the regional biodiversity (De Waele 2003), yet their ecological aspects have been almost completely neglected. This study was conducted at Sa Vena spring (Su Gologone karst system). Our aims were to (1) characterize epiphytic and epilithic diatom assemblages on a complete seasonal cycle, (2) describe their relationships with environmental variables, and (3) assess variations in the assemblages over

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time. Additionally, we assessed the effects of the flash flood caused by the extratropical Cyclone Cleopatra (locally, but later renamed Ruven by the Institut für Meteorologie der FU Berlin) in November 2013. We hypothesized significant differences between diatom assemblages on cobbles and macrophytes and no significant differences between diatom assemblages on a single substrate over time, despite the occurrence of the flash flood. In fact, springs are places of low population change and long-term stability (Gerecke et al. 2011).

Study site

Sa Vena (Fig. 1) is the smaller spring of the Su Gologone karst system in central-eastern Sardinia, Italy (Bianco 1993). The spring system was recognized as both a Natural Monument (R.D. 845/1998) and a Zone of Respect (L.D. 152/1999). It is within the Gennargentu and the Gulf of Orosei National Park (D.P.R. 30 March 1998), the Site of Community Importance and the Zone of Special Protection ITB022212 for the biogeographic Mediterranean region.

Sa Vena is a permanent rheocrenic spring that flows at 103.7 m a.s.l. The main disturbances are sheep breeding, intensive animal farming in the recharge area, and tourism (De Waele 2009). The water is used for local drinking purposes, with an uptake system of ~100 L s^{-1} (Bianco 1993). The spring undergoes periodic floods due to the overflow of the Pedra ‘e Othoni dam on the Cedrino River after intense rainfall (De Waele 2009). The extratropical Cyclone Cleopatra hit the western Mediterranean basin, including Sardinia, between 18 and 19 November 2013 (Supplemental Photo 1), resulting in loss of human life and extensive damage in many areas. The cyclone caused an extreme flash flood at the Sa Vena spring (Supplemental Photo 2), making it inaccessible and interrupting the water supply for several months.

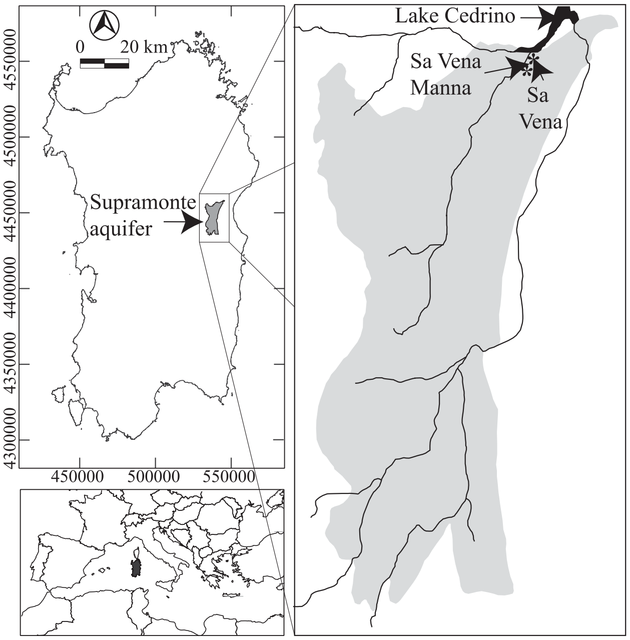

Figure 1. Geographic location of the Sa Vena spring (Su Gologone karst system, Sardinia, Italy).

Methods

Sampling

Water samples for physical and chemical analyses were collected monthly from January 2014 to April 2015 using 1 L polyethylene bottles. Benthic diatoms were collected once each season in 2014, with 2 additional samples in winter and spring 2015. Epilithic diatoms were collected by scraping 5 cobbles free of macroscopic filamentous algae and other plant cover with a hard-bristled toothbrush. Epiphytic diatoms were collected from macrophytes by squeezing submerged portions of *Nasturtium officinale* R. Brown, *Adiantum capillus-veneris* Linnaeus, *Polypodium vulgare* Linnaeus, and *Brachythecium rivulare* Schimper (ISPRA 2007). The material was integrated into a composite sample. All diatom samples were preserved in 100 mL polyethylene bottles and fixed *in situ* with formaldehyde (final concentration: 4% by volume).

Measurements and analyses

Temperature (T), pH, conductivity (Cond), and oxygen saturation (DO%) were measured *in situ* with a multiparameter probe (YSI ProPlus). Alkalinity (Alk), dissolved oxygen (DO), chlorides (Cl^-), total hardness (THar), biological oxygen demand (BOD_5), suspended solids (TSS), and concentrations of calcium (Ca^{2+}), magnesium (Mg^{2+}), iron (Fe^{2+}), and manganese (Mn^{2+}) were analysed in laboratory using methods of IRSA-CNR (1994), and nutrients and reactive silica (RSi) were analyzed using methods of Strickland and Parsons (1972).

Diatom subsamples were treated with hydrogen peroxide (30% v/v) on a heating plate, and diluted hydrochloric acid (37%) was added on the cooled samples to remove carbonates (ISPRA 2007). Permanent microscope slides were mounted using Styrax resin (refractive index = 1.59). Diatom analysis was performed by a Zeiss light microscope at 1000× magnification in 2 consecutive steps: (1) general screening of slides to create a complete floristic list, and (2) counting of at least 400 valves and/or frustules. Species identification was made mainly according to Krammer and Lange-Bertalot (1986, 1988, 1991a, 1991b) and Lange-Bertalot et al. (2003).

Data processing and statistical analyses

Autecological preferences of diatoms were attributed according to Dell'Uomo (2004) and Van Dam et al. (1994). The German Red List (Lange-Bertalot 1996) was used as the only international reference available for the vulnerability of species. The structure of assemblages was evaluated by species richness using the Shannon-Wiener diversity index (H'; Shannon and Weaver 1949) and Pielou evenness index (J'; Pielou 1975), calculated by OMNIDIA 7 version 8.1 software (Lecointe et al. 1993). Statistical analyses were performed on environmental and log $(x + 1)$ transformed diatom data. We considered all species with relative abundance (RA) $\geq$1.5% in at least one sample.

The similarity between epiphytic and epilithic assemblages was analysed by non-metric multidimensional scaling (nMDS) ordination and a one-way analysis of similarities (ANOSIM). The percent contribution of each species to the differences between the 2 groups was determined by SIMPER analysis.

Relationships between diatoms and environmental variables were investigated by redundancy analyses (RDA) performed on the 2 substrates separately. The significance of variables was tested using Monte Carlo simulations with 499 permutations.

Seasonal differences in the composition of epiphytic or epilithic assemblages and diversity as expressed by Shannon-Wiener's index were explored by the Kolmogorov-Smirnov test and the Hutcheson *t*-test. These tests were also used to assess changes induced by the flash flood comparing 2 consecutive winters (2014 vs. 2015) and springs (2014 vs. 2015; i.e., immediately following the extreme event and 1 year later). The epilithic diatom data were integrated with data recorded in 2010–2011 (Lai et al. 2016). In this case, the 2 tests were run including 3 winter samples (Dec 2010, Jan 2014, and Jan 2015). An nMDS was also performed on the environmental variables T, pH, Cond, DO%, Alk, Cl^-, THar, BOD_5, TSS, Ca^{2+}, Mg^{2+}, and nutrients (2010–2011 vs. 2014–2015; i.e., before and after the flash flood event). The significance of the differences was determined by ANOSIM and the percent contribution of each variable to the differences by SIMPER.

The nMDS, ANOSIM and SIMPER were performed by PRIMER 5 (Clarke and Gorley 2001), Kolmogorov-Smirnov test, and Hutcheson *t*-test by R 3.2.2 (Venables et al. 2016), and RDA by Canoco 4.5 (ter Braak and Šmilauer 2002).

Results

Environmental variables

Results of physical and chemical variables (Table 1) show that T ranged from 11.5 °C in January to 14.5 °C in May. The DO% was generally $\geq$75%, and the pH range was 7.3–8.3. Cond ranged between 241 and 379 $\mu S\ cm^{-1}$, and the THar measured as calcium carbonate ($CaCO_3$) was 135–175 mg L^{-1}. The content of Cl^- was 10.6–24.8 mg L^{-1}, and Ca^{2+} (27.5–54.0 mg L^{-1}) was more abundant than Mg^{2+} (6.1–21.9 mg L^{-1}). BOD_5 ranged from 0.8 to 7.4 mg L^{-1}, with peaks during some months. TSS was maximum (2.9 mg L^{-1}) in May. The phosphate (PO_4-P) values ranged between 5 µg L^{-1} (Nov) and 13 µg L^{-1} (Aug). Total P (TP) values were highest in January and February 2014 (~60 µg L^{-1}). Nitrate (NO_3-N), with an annual mean value of 614 µg L^{-1}, provided the greatest contribution to total nitrogen (TN), which ranged from 493 µg L^{-1} (May) to 2490 µg L^{-1} (Aug).

Diatom assemblages

We identified 151 taxa (58 genera), of which 92 (37 genera) were present in the counts (Supplemental Table). Epiphytic assemblages were composed of 78 taxa (35 genera), with 8 taxa abundant, 6 taxa frequent, and 64 taxa rare. The dominant species were *Planothidium lanceolatum* (RA = 18.4%), *Achnanthidium subatomus* (RA = 12.9%), and *Meridion circulare* (RA = 8.2%). Epilithic assemblages were composed of 73 taxa (32 genera), with 4 taxa abundant, 9 taxa frequent, and 60 taxa rare. The dominant species were *Achnanthidium minutissimum* (RA = 19.6%), *A. subatomus* (RA = 16.4%), and *Amphora pediculus* (RA = 15.0%).

Autecological preferences highlighted the prevalence of species alkaliphilous (78%), halophobous and oligohalobous exigent (50%), xenosaprobic and oligosaprobic (53%), characteristic of hypotrophic and oligotrophic waters (41%), and bound to the aquatic environment (85%).

Overall, 6 taxa (7% of the taxa present in the counts) are threatened according to the Red List: *Achnanthidium lineare* "endangered"; *Ulnaria biceps* "at risk"; *Cocconeis neothumensis*, *Encyonema neomesianum*, and *Nitzschia solgensis* "in regression"; and *Diploneis minuta* "extremely rare."

The results of the biotic integrity indices (Table 2) show that species richness varied from 28 to 49 in the epiphytic assemblages and from 37 to 47 in the epilithic assemblages. The Shannon-Wiener diversity index ranged from 3.38 to 4.48 in the epiphytic assemblages and from 3.50 to 4.09 in the epilithic assemblages. The Pielou

Table 1. Monthly, mean, standard deviation, minimum, and maximum values of the physical and chemical parameters recorded in the field and analysed in the laboratory (D.L. = detection level).

Variables	21 Jan 2014	26 Feb 2014	26 Mar 2014	14 Apr 2014	21 May 2014	18 Jun 2014	21 Jul 2014	25 Aug 2014	29 Sep 2014	20 Oct 2014	19 Nov 2014	17 Dec 2014	14 Jan 2015	02 Mar 2015	23 Apr 2015	Mean (SD)	Min–max
Temperature (°C)	11.5	12.0	12.5	12.7	14.5	13.0	12.9	13.1	13.0	13.0	13.0	13.2	13.2	13.0	13.2	12.9 (1.2)	11.5–14.5
pH (units)	8.2	8.3	7.6	7.3	7.8	8.2	7.4	7.5	7.5	7.4	7.4	7.3	7.5	7.9	7.5	7.7 (0.5)	7.3–8.3
Conductivity (µS cm^{-1})	243	241	303	322	332	339	350	366	379	376	373	358	363	310	308	331 (46.0)	241–379
Alkalinity (meq L^{-1})	2.4	2.4	2.5	2.5	2.9	3.0	3.1	3.2	3.1	3.3	3.3	3.1	3.3	3.0	2.5	2.9 (0.1)	2.4–3.3
Dissoved oxygen (mg L^{-1})	9.2	9.5	10.6	10.8	6.5	8.3	9.8	9.7	10.4	10.2	6.8	6.6	10.8	10.9	9.9	9.3 (0.5)	6.5–10.9
Oxygen saturation (%)	84	88	88	90	63	79	93	92	99	97	64	63	103	104	95	87 (7.8)	63–104
BOD_5 (mg L^{-1})	4.6	4.4	2.7	5.2	4.2	6.5	1.4	4.2	0.8	3.8	1.7	7.4	1.5	3.7	1.0	3.5 (2.5)	0.8–7.4
Cl^- (mg L^{-1})	16.0	15.6	15.0	14.2	10.6	—	14.2	17.7	21.3	23.0	17.5	23.0	24.8	24.8	21.3	18.5 (3.8)	10.6–24.8
$CaCO_3$ (mg L^{-1})	135	138	140	145	175	—	145	174	175	175	174	175	160	135	170	158 (24.7)	135–175
$P\text{-}PO_4^{3-}$ (µg L^{-1})	10	7	9	9	10	9	7	13	11	7	5	8	8	7	7	9 (2)	5–13
TP (µg L^{-1})	64	62	11	10	—	18	29	35	22	8	9	15	9	7	9	22 (39)	7–64
NH_4-N (µg L^{-1})	36	< D.L.	7	30	6	11	< D.L.	< D.L.	20	34	< D.L.	23	12	19	5	19 (22)	< D.L.–36
NO_2-N (µg L^{-1})	1	1	< D.L.	2	5	1	1	1	< D.L.	1	11	< D.L.	< D.L.	1	2	3 (1)	< D.L.–311
NO_3-N (µg L^{-1})	594	488	440	457	521	606	664	680	677	719	484	609	952	754	569	614 (18)	< D.L.–952
TN (µg L^{-1})	658	1925	997	1022	493	768	1089	2490	-	1853	741	951	1492	1481	898	1204 (170)	493–2490
RSi (mg L^{-1})	2.1	2.0	1.9	1.9	2.0	2.0	2.0	2.0	2.0	2.0	1.9	1.9	1.7	1.6	2.0	1.9 (0)	1.6–2.1
Ca^{2+} (mg L^{-1})	42.0	43.0	45.0	48.0	46.0	—	46.0	53.5	54.0	52.0	52.7	52.0	28.0	27.5	36.0	44.7 (4.2)	27.5–54.0
Mg^{2+} (mg L^{-1})	7.3	7.2	6.9	6.1	14.6	—	14.6	9.7	9.7	11.0	9.7	11.0	21.9	19.4	19.4	12 (8.6)	6.1–21.9
TSS (mg L^{-1})	0.5	0.4	0.6	< D.L.	2.9	0.2	0.2	0.4	1.4	< D.L.	< D.L.	< D.L.	< D.L.	< D.L.	0.2	0.8 (0.2)	0.2–2.9

Table 2. Seasonal values of species richness, Shannon-Wiener diversity index (H′), and Pielou index (J′) for evenness for epiphytic and epilithic diatom assemblages.

	Richness		Diversity index (H′)		Pielou index (J′)	
	epiphytic	epilithic	epiphytic	epilithic	epiphytic	epilithic
WIN-14	49	39	4.48	3.93	0.80	0.74
SPR-14	41	41	3.88	3.94	0.72	0.74
SUM-14	42	47	3.80	4.09	0.70	0.74
AUT-14	28	41	3.38	3.66	0.70	0.68
WIN-15	42	41	4.34	3.54	0.80	0.66
SPR-15	35	37	3.61	3.50	0.70	0.67

index (evenness) showed a minimum of 0.70 and a maximum of 0.80 in the epiphytic assemblages, and a minimum of 0.66 and a maximum of 0.74 in the epilithic assemblages.

Differences between assemblages and relationships with environmental variables

Epiphytic and epilithic assemblages were clearly separated in the nMDS ordination (Fig. 2). The ANOSIM test confirmed significant differences (global $R = 0.754$, $p = 0.002$).

The greatest contribution to the differences was attributed to *Achnanthidium minutissimum* and *Planothidium lanceolatum*, dominant on cobbles and macrophytes, respectively, by SIMPER analysis (Table 3). A higher similarity was observed in the epilithic assemblages (67.30%) than in the epiphytic assemblages (59.89%).

The Kolmogorov-Smirnov test and the Hutcheson *t*-test revealed significant differences in the species composition and diversity of the epiphytic assemblages in winter, spring, and autumn on annual and interannual scales (Table 4). Significant differences were also found between epilithic assemblages by the Hutcheson *t*-test, comparing spring and winter data on a multiannual scale (Table 4).

A clear separation of environmental data among years (2010-2011 vs. 2014–2015) was highlighted by nMDS (Fig. 3). Significant differences before and after the extreme flash flood were confirmed by the ANOSIM test (global $R = 0.207$, $p = 0.001$). Cond, THard, DO%, and Ca^{2+} contributed most to these differences (cumulative percent contribution = 79.71%) according to the SIMPER analysis (Table 5).

RDA showed associations between species and environmental variables both for epiphytic and epilithic assemblages. RDA for the epiphytic assemblages explained 64.1% of the variance in the 2 first axes (axis 1: 37.6% and axis 2: 26.5%). Species like *C. placentula* and *C. euglypta* were associated with Alk and significantly explained the total variability in the species composition ($p = 0.002$; Fig. 4a). RDA for the epilithic diatom assemblages explained 52.1% of the variance in the 2 first axes (axis 1: 33.4% and axis 2: 18.7%). Species like

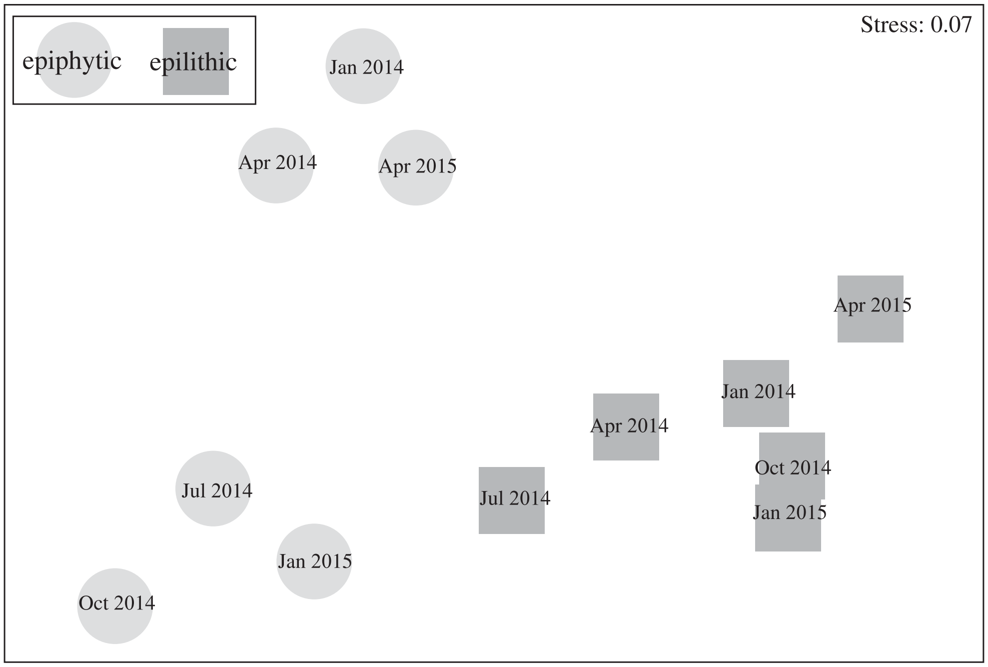

Figure 2. Non-metric multidimensional scaling (nMDS) for epiphytic and epilithic diatoms collected in 2014 and 2015.

Table 3. Contribution of each taxon (with relative code) to differences found with the nMDS analysis between the 2 groups of epiphytic and epilithic assemblages according to SIMPER test.

Taxon (code)	Contribution (%)	Cumulate contribution (%)
Achnanthidium minutissimum (ADMI)	18.8	18.8
Planothidium lanceolatum (PTLA)	12.5	31.3
Amphora pediculus (APED)	7.6	38.8
Meridion circulare (MCIR)	7.2	46.1
Cocconeis placentula (CPLA)	6.1	52.2
Diatoma mesodon (DMES)	6.0	58.2
Cocconeis euglypta (CEUG)	5.7	63.8
Nitzschia fonticola (NFON)	5.0	68.8
Achnanthidium subatomus (ADSU)	3.4	72.2

C. euglypta and *C. placentula* were associated with DO% and significantly explained the total variability in the species composition ($p = 0.004$; Fig. 4b).

Discussion

Species composition and structure of epiphytic and epilithic diatom assemblages

The karst spring hosts a rich and diversified diatom flora despite its small size. This small size makes it vulnerable to anthropogenic disturbances, such as water abstraction, which can alter the morphology and cause an impoverishment of the biota (e.g., Cantonati et al. 2012). The diatom flora is composed of some crenophilous taxa, such as *Eunotia minor* and *Odontidium hyemale* (rare) and *O. mesodon* and *M. circulare* (abundant), also found in springs in the Alps (e.g., Falasco et al. 2012) and in Bosnia and Herzegovina (Dedić et al. 2015). The presence of *O. hyemale* is interesting because this species is typically found in the Nordic-Alpine diatom flora (Dell'Uomo 1990). Other taxa, not bound to the aquatic environment and threatened according to the Red List, such as *Diploneis minuta* and *Nitzschia solgensis*, highlight the role of the spring as ecotone and the importance of preserving this biotope.

Epilithic and epiphytic assemblages showed significant differences, mainly attributable to *Achnanthidium minutissimum* and *Planothidium lanceolatum*. In our study, they were most abundant on cobbles (winter-spring) and on macrophytes (summer-autumn), respectively, suggesting a role of the hydrologic regime on diatom community composition.

Epiphytic assemblages showed highest species richness, diversity, and evenness, suggesting a high heterogeneity at the microhabitat scale. Among the macrophytes, the mosses in particular form an ecotone between terrestrial and aquatic conditions, creating a wide array of microhabitats (e.g., Bottazzi et al. 2011). Similar results were reported for Alpine rheocrenic springs (e.g., Gesierich and Kofler 2010). Epiphytic assemblages also showed lower similarity in their species composition than epilithic assemblages, suggesting macrophytes are less stable substrates than cobbles for diatoms and may be more susceptible to seasonal hydrological variations and water abstraction.

Relationships of diatoms with environmental variables

The diatom assemblages reflected the calcareous nature of the geological substrate and the water chemistry, confirming the results of our previous study (Lai et al. 2016). Alkalinity and DO% explained significant amount of variance in epiphytic and epilithic diatom assemblages, respectively. Alkalinity is a key parameter in determining the species composition (Pan and Stevenson 1996) and a main driver for diatom communities in lotic systems (e.g., O'Driscoll et al. 2012). Alkalinity can be

Table 4. Results of Kolmogorov-Smirnov test and Hutcheson *t*-test values comparing seasonal species composition, and diversity as expressed by the Shannon-Wiener's index on annual and multiannual scales for epiphytic and epilithic diatom assemblages. D = statistical test measuring the maximum absolute difference between cumulative distribution functions of species composition; *p* (same) = level of significance; *t* = statistical test comparing the diversity of 2 community samples as expressed by the Shannon-Wiener's index; *p* = level of significance. Significant values ($p < 0.05$) are reported in bold.

			Kolmogorov-Smirnof test				Hutcheson *t*-test			
			Epiphytic		Epilithic		Epiphytic		Epilithic	
			D:	*p* (same):	D:	*p* (same):	*t*	*p*	*t*	*p*
WIN 2014	vs.	SPRI 2014	**0.3077**	**0.0009**	0.1370	0.4706	**3.6095**	**0.0004**	−0.8377	0.4035
SPR 2014	vs.	SUM 2014	0.1282	0.5147	0.0822	0.9587	0.2831	0.7775	1.1507	0.2516
SUM 2014	vs.	AUT 2014	0.1667	0.2078	0.2329	0.0318	1.0636	0.2891	1.6537	0.1001
AUT 2014	vs.	WIN 2015	0.1511	0.1912	0.1370	0.4706	**−3.9577**	**0.0001**	−0.8544	0.3942
WIN 2015	vs.	SPR 2015	**0.2692**	**0.0054**	0.1314	0.3734	**3.3207**	**0.0011**	1.0313	0.3039
WIN 2010	vs.	WIN 2014	—	—	0.1440	0.4801	—	—	**−2.0534**	**0.0415**
WIN 2010	vs.	WIN 2015	—	—	0.1373	0.4808	—	—	−0.8374	0.4036
WIN 2014	vs.	WIN 2015	**0.2436**	**0.0159**	0.1096	0.7487	1.0917	0.2766	1.1743	0.2419
SPR 2011	vs.	SPR 2014	—	—	0.1233	0.6072	—	—	−1.6063	0.1101
SPR 2011	vs.	SPR 2015	—	—	0.0822	0.9587	—	—	1.7499	0.0820
SPR 2014	vs.	SPR 2015	**0.2436**	**0.0159**	0.1644	0.2534	0.7119	0.4775	**2.2368**	**0.0266**

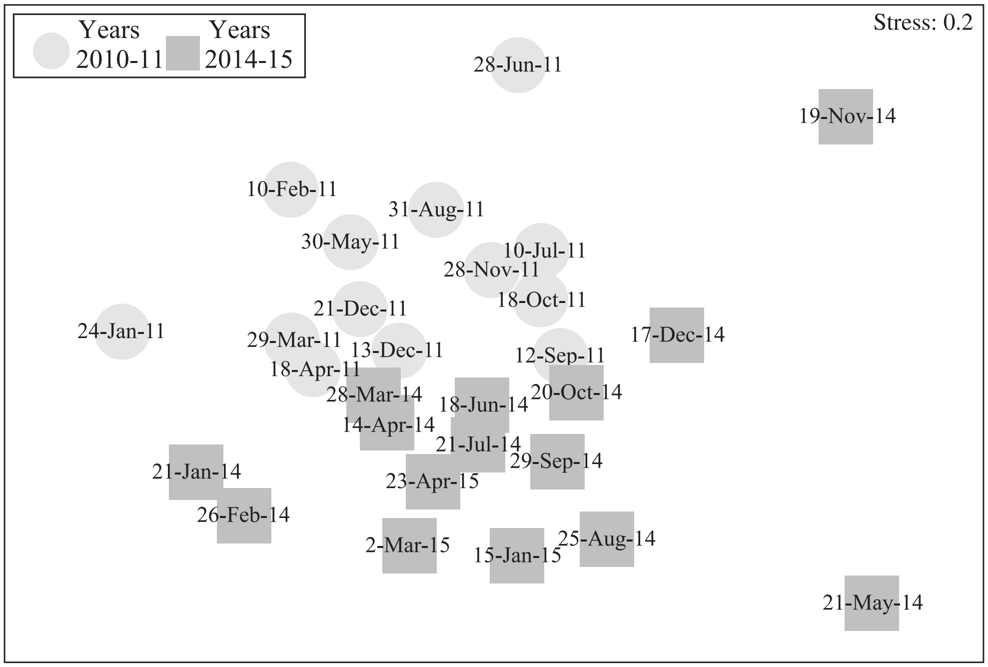

Figure 3. Non-metric multidimensional scaling (nMDS) of the environmental variables recorded in 2010–2011 and 2014–2015 (i.e., before and after the flash flood event due to Cyclone Cleopatra).

affected by many factors, including water levels (Salman et al. 2014). DO is the result of several physical and biological processes of lotic systems (Betina and Frank 1993) and can be affected by many factors, including organic matter (Maitland 1990). In our study, BOD_5, which negatively correlated with DO, can be the most probable driver of epilithic diatoms. Other studies reported BOD_5 among the influencing factors of diatom communities in running waters (e.g., Blanco et al. 2008).

Variation of diatom assemblages over time

Significant differences in the composition and diversity of epiphytic diatom assemblages occurred both on annual (2014) and interannual scales (2014–2015). On the annual scale, significant differences were observed in winter versus spring. Species richness, diversity, and evenness were higher in winter. The higher flow rate may have favored the development of mosses, with a greater availability of microhabitats for diatoms. In fact, mosses prefer fast and turbulent waters (Haury et al. 2000). Significant differences were also found at the interannual scale (between winters, springs, and in autumn vs. winter), indicating the importance of hydrologic regime and the impact of the flash flood on the communities. Comparison of the winter assemblages (2014 vs. 2015), immediately following and 1 year after the flash flood, revealed differences in presence/absence and in relative abundance of some taxa with different growth forms. In general, short stature taxa tightly adhered, adnate, and prostrate, such as *Achnanthidium minutissimum*, *Amphora indistincta*, *Cocconeis* spp., and *Planothidium lanceolatum*, were less abundant in winter 2014 than in winter 2015. They are considered to be resistant to high current velocities and may be resistant to flash flood events (Rimet and Bouchez 2012). Other taxa such as *Navicula cryptotenella*, *N. tripunctata*, *Nitzschia fonticola*, and *N. dissipata* considered fast-moving and adapted to turbulent environments (Rimet and Bouchez 2012) showed higher abundance in winter 2014. *Ellerbeckia arenaria*, a filamentous taxon that prefers low current velocities, was not observed in winter 2014 but appeared in winter 2015. Similar results were reported in lotic systems in different geographic areas (e.g., Tornés et al. 2015). The epilithic diatom assemblages showed no significant differences in their composition on annual and multiannual scales, revealing a greater resistance to the hydrologic regime and flash flood event than the epiphytic assemblages. Similar responses were reported for epilithic diatoms of small rivers in the Czech Republic (Adámek et al. 2016).

Table 5. Contribution of the environmental variables to differences found with the nMDS analysis between the periods 2010–2011 (before the flash flood event) and 2014–2015 (after the flash flood event) according to SIMPER test.

Variables	Contribution (%)	Cumulate contribution (%)
Conductivity (µS cm^{-1})	38.0	38.0
Total hardness as $CaCO_3$ (mg L^{-1})	19.3	57.4
Oxygen (% saturation)	12.3	69.7
Ca^{2+} (mg L^{-1})	10.0	79.7

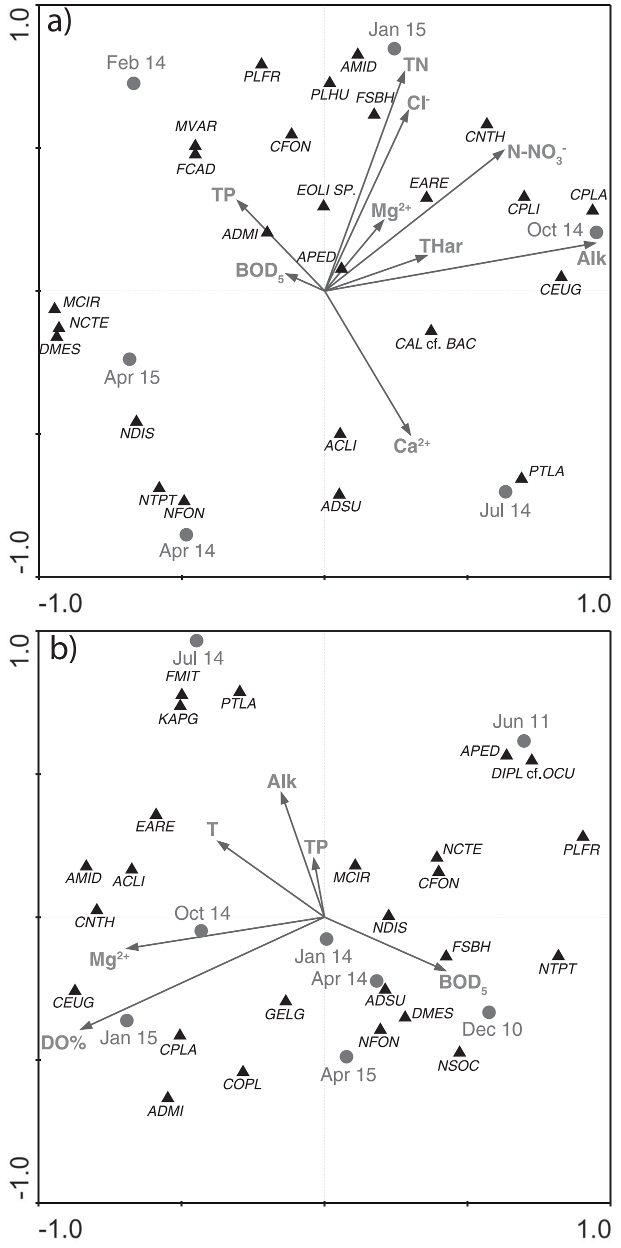

Figure 4. Biplot of RDA analysis for (a) epiphytic diatom assemblages and (b) epilithic diatom assemblages. Environmental variables (vectors), diatom taxa (triangles), and samplings (points). All taxa are reported with the respective codes of OMNIDIA software. ACLI = *Achnanthidium lineare*; ADMI = *Achnanthidium minutissimum*; ADSU = *Achnanthidium subatomus*; AMID = *Amphora indistincta*; APED = *Amphora pediculus*; CALO cf. BAC = *Caloneis* cf. *bacillum*; CEUG = *Cocconeis euglypta*; CFON = *Caloneis fontinalis*; CNTH = *Cocconeis neothumensis*; COPL = *Cocconeis pseudolineata*; CPLA = *Cocconeis placentula*; CPLI = *Cocconeis lineata*; DMES = *Odontium mesodon*; DIPL cf. OCU = *Diploneis* cf. *oculata*; EARE = *Ellerbeckia arenaria*; EOLI sp. = *Eolimna* sp.; FCAD = *Fragilaria candidagilae*; FMIT = *Fallacia mitis*; FSBH = *Fallacia subhamulata*; GELG = *Gomphonema elegantissimum*; KAPG = *Kolbesia gessneri*; MVAR = *Melosira varians*; MCIR = *Meridion circulare*; NCTE = *Navicula cryptotenella*; NDIS = *Nitzschia dissipata*; NFON = *Nitzschia fonticola*; NSOC = *Nitzschia sociabilis*; NTPT = *Navicula tripunctata*; PLFR = *Planothidium frequentissimum*; PLHU = *Platessa hustedtii*; PTLA = *Planothidium lanceolatum*.

In our study, significant differences were found in the diversity of assemblages on a multiannual scale. Higher values were found in winter and spring 2014 just after the flash flood, and lower values were found in winter 2010 and spring 2015 before and after the flash flood, respectively. These results indicated high community resilience and a return to predisturbance conditions, with low diversity occurring while the spring recovered and returned to the normal seasonal hydrologic regime.

Conclusions

Sa Vena spring was characterized by a high species richness and biotic integrity despite some direct impacts (e.g., water abstraction). The diatom assemblages showed significant differences between the 2 substrates (according to our initial hypothesis) and on the same substrate (contrary to our initial hypothesis), mainly due to seasonal variations in water level (irregular rainfall, water abstraction, and periodic floods). The extreme flash flood induced changes in environmental conditions and mainly affected the epiphytic assemblages. Epiphytic diatoms are highly sensitive to temporal variations in water level and they can highlight changes due to the hydrologic disturbance in spring environments. The results suggest the importance of the proper management and preservation of this biotope, both for the high biodiversity and potential vulnerability to climate change.

Trampling due to tourist turnout and a greater water abstraction could create a depletion of the present microhabitats, and consequently of the biodiversity over time. This study contributes to knowledge of the diatom microflora and ecological aspects in karst springs of Sardinia, which remain little studied to date. It provides first data on the species composition at small spatial and temporal scales, which may be useful for future comparisons with other springs of the same type in the Island and Mediterranean area.

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We thank Irene Ara, Giommaria Canu, and Giampaolo Dore for their support in the field and Bastianina Manca, Pasqualina Farina, and Tiziana Caddeo for the laboratory analyses. We are also grateful to Malvina Urbani for the identification of *Brachythecium rivulare*.

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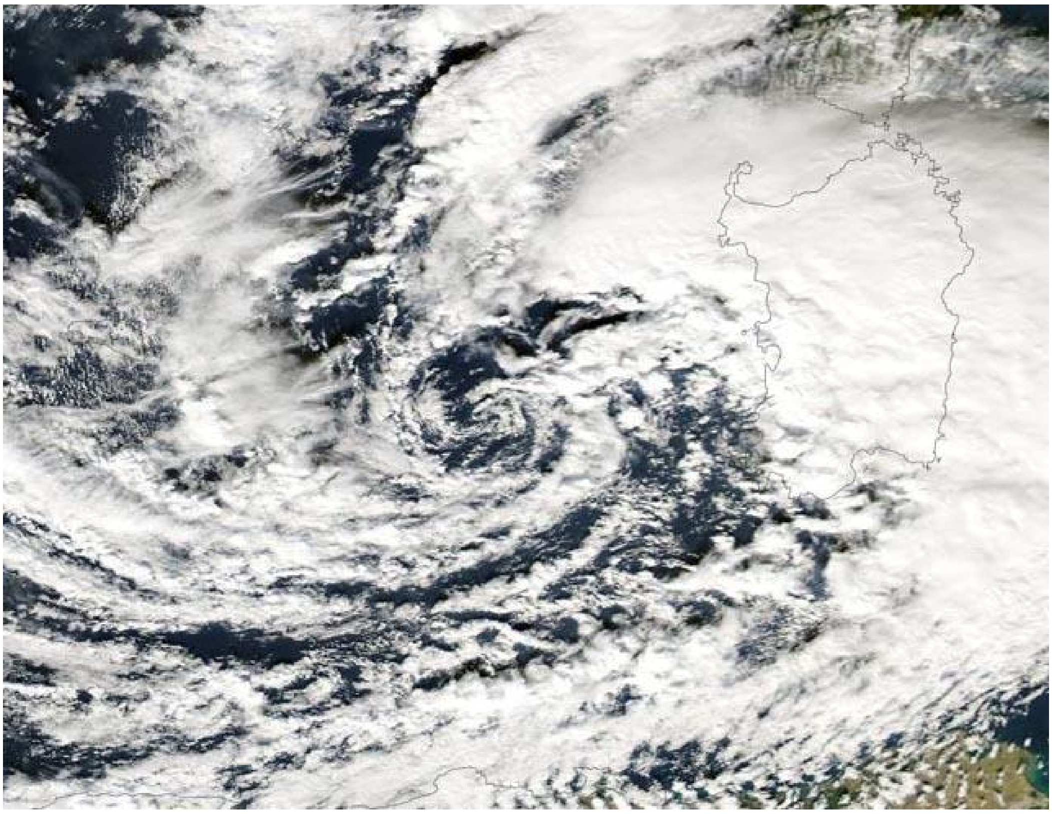

Supplementary Photo 1. Extratropical Cleopatra Cyclone that hit Sardinia in November 18, 2013. Source: TW (Researcher, journalist and editor of The Watchers). http://watchers.new/2013/11/19/cyclone-cleopatra-sardinia-record-breaking-rainfall november-2013/.

Supplementary Photo 2. Area around the spring before and almost a month after (December 15, 2013) the flash flood event occurred with the Extratropical Cleopatra Cyclone (November 18, 2013).

Supplementary Table. List of the diatom taxa observed in the Sa Vena spring (Su Gologone karst system) that contributed ≥1.5% to total abundance in at least one sample and their ecological preferences according to Dell'Uomo (2004), Torrisi and Dell'Uomo (2009) and Van Dam et al. (1994).

Abundance categories: a = abundant (>5%); f = frequent (1.5–5%); r = rare (<1.5%).

Preferences for **pH**: akb = alkalibiontic; ak = alkaliphilous, n = circumneutral; c = acidophilous; i = indifferent; preferences for **Salinity**: hb = halophobous, oe = oligohalobous exigent, ot = oligohalobous tolerant, h = halophilous; preferences for **Saprobity**: x = xenosaprobic, o = oligosaprobic, β = β-mesosaprobic, α = α-mesosaprobic; p = polysaprobic, preferences for **Trophic state** (species characteristic of): hypo = hypotrophic environments, oligo = oligotrophic environments, meso = mesotrophic environments, eu = eutrophic environments; preferences for **Moisture**: 1= never, or only very rarely, occurring outside water bodies, 2 = mainly occurring in water bodies, sometimes on wet places, 3 = mainly occurring in water bodies, also rather regularly on wet and moist places, 4 = mainly occurring on wet and moist or temporarily dry places, 5 = nearly exclusively occurring outside water bodies. **Red List** = Germain Red List of diatom taxa (Lange-Bertalot 1996): * = currently not endangered, ** = surely not endangered, 2 = highly endangered, 3 = endangered, D = insufficient data, G = at risk, V= in regression, R = extremely rare. For all systems: - = no data.

Taxon	Abundance categories		pH	Salinity	Saprobity	Trophic state	Moisture	Red List
	Cobbles	Macrophytes						
Achnanthidium lineare W. Smith	r	r	n	-	-	-	-	3
Achnanthidium minutissimum (Kützing) Czarnecki	a	f	n-ak	oe	o	oligo	3	
Achnanthidium subatomus (Hustedt) Lange-Bertalot	a	a	-	-	-	-	-	*
Amphipleura pellucida (Kützing) Kützing	r	r	ak	oe	o-β	oligo-meso	2	*
Amphora indistincta Levkov	f	f	-	-	-	-	-	-
Amphora meridionalis Levkov	r	r	-	-	-	-	-	-
Amphora ovalis (Kützing) Kützing	r	r	ak	ot	o-β	oligo-meso	1	**
Amphora pediculus (Kützing) Grunow in Schmidt et al.	a	a	ak	ot	x-β	oligo-meso	3	**
Amphora sp.	r							
Amphora vetula Levkov	r		-	-	-	-	-	-
Caloneis cf. *bacillum*	r	r						
Caloneis fontinalis (Grunow) Cleve-Euler	f	r	-	-	-	-	-	-
Caloneis lancettula (Schulz) Lange-Bertalot & Witkowski	r	r	-	-	-	-	-	-
Cocconeis euglypta Ehrenberg	r	a	ak	ot-h	o-α	oligo-eu	2	**
Cocconeis lineata Ehrenberg	r	r	ak	ot	x-β	oligo-meso	2	**
Cocconeis neothumensis Krammer	r	f	akb	-	o	-	-	V
Cocconeis pediculus Ehrenberg		r	ak	ot-h	β	eu	1	**
Cocconeis placentula Ehrenberg	f	a	ak	ot	x-β	oligo-meso	2	
Cocconeis pseudolineata (Geitler) Lange-Bertalot	r	r	ak	ot	x-β	oligo-meso	-	D
Denticula tenuis Kützing	r	r	ak	hb	o	oligo-meso	3	*
Diploneis cf. *oculata*	r	r						
Diploneis elliptica (Kützing) Cleve		r	ak	oe	x-o	hypo-oligo	3	
Diploneis minuta J.B. Petersen		r	-	-	-	-	5	R
Diploneis separanda Lange-Bertalot	r	r	-	-	-	-	-	-
Diploneis sp. 1	r	r						
Diploneis sp. 2		r						
Ellerbeckia arenaria (Moore ex Ralfs) R.M. Crawford	r	r	ak	hb	o	hypo	4	**
Encyonema neomesianum Krammer	r		ak	-	-	-	-	V
Encyonema silesiacum (Bleisch) D.G. Mann	r	r	n	ot	β	meso	1	*
Eolimna sp.	r	r						
Eolimna minima (Grunow) Lange-Bertalot	r	r	ak	h	α	eu	3	**
Eunotia minor (Kützing) Grunow	r	r	ac	-	o	-	4	*
Eunotia pectinalis (Kützing) Rabenhorst	r		ac	hb-oe	x-o	hypo-oligo	3	
Eunotia sp.	r	r						
Fallacia mitis (Hustedt) D.G. Mann	r	r	ak	oe	x	oligo	-	-
Fallacia subhamulata (Grunow) D.G. Mann		r	n	oe	o-β	meso	3	*
Fragilaria capucina Desmazières	r	r	n-ak	oe	o	oligo	-	
Fragilaria candidagilae Almeida, C. Delgado, Novais & S. Blanco in Delgado et al.		r	n	oe	o-β	meso	-	-
Fragilaria sp.	r	r						
Fragilaria vaucheriae (Kützing) J.B. Petersen		r	ak	ot	α	eu	3	**
Frustulia vulgaris (Thwaites) De Toni		r	ak	oe	o-β	meso	3	**
Geissleria decussis (Østrup) Lange-Bertalot & Metzeltin	r	r	ak	oe	o	oligo	3	**
Gomphonema clavatum Ehrenberg	r	r	n-i	hb	x-o	oligo	2	*
Gomphonema elegantissimum E. Reichardt & Lange-Bertalot	r	r	-	-	-	-	-	-

Taxon	Abundance categories		pH	Salinity	Saprobity	Trophic state	Moisture	Red List
	Cobbles	Macrophytes						
Gomphonema micropus Kützing	r	r	ak	α-meso	β	eu	-	*
Gomphonema sp.	r							
Gomphonema truncatum Ehrenberg		r	ak	ot	o-β	oligo-meso	2	*
Gomphosphenia grovei var. *lingulata* (Hustedt) Lange-Bertalot	r		-	-	-	-	-	-
Halamphora montana (Krasske) Levkov		r	ak	oe	o-β	meso	4	*
Halamphora veneta (Kützing) Levkov		r	-	-	-	-	-	
Humidophila contenta R.L. Lowe, Kociolek, J.R. Johansen, Van de Vijver, Lange-Bertalot	r	r	ak	oe-ot	o-β	oligo-meso	4	**
Iconella helvetica (Brun) Ruck & Nakov		r	ac	oe	o	oligo	3	-
Karayevia clevei (Grunow) Bukhtiyarova	r	r	ak	oe	o-β	oligo-meso	1	
Kolbesia gessneri (Hustedt) Aboal	r	r	-	-	-	-	-	-
Luticola goeppertiana (Bleisch) D.G. Mann	r		ak	hb	α-poli	eu-hyper	3	**
Melosira varians C. Agardh	r	f	ak	ot	x-α	oligo-eu	2	**
Meridion circulare (Greville) C. Agardh	f	a	ak	hb-oe	x-o	oligo	1	
Navicula antonii Lange-Bertalot	r	r	-	-	-	-	-	**
Navicula capitatoradiata H. Germain	r	r	ak	ot-h	α	eu	1	**
Navicula cincta (Ehrenberg) Ralfs	r		ak	oe	o-β	oligo-eu	-	**
Navicula cryptotenella Lange-Bertalot	f	f	ak	oe	o-β	oligo-meso	2	-
Navicula cryptotenelloides Lange-Bertalot	r	r	ak	-	-	-	-	**
Navicula gregaria Donkin	r	r	ak	h	α	meso	3	**
Navicula tenelloides Hustedt	r	r	ak	h	o	eu	4	*
Navicula tripunctata (O.F. Müller) Bory	f	f	ak	hb-oe	o	oligo	3	**
Navicula veneta Kützing	r		ak	hb	α-poli	eu-hyper	3	**
Navicula rostellata Kützing	r	r	ak	ot	β-α	meso-eu	1	**
Nitzschia frustulum (Kützing) Grunow		r	ak	ot-h	β-α	meso-eu	3	
Nitzschia commutata Grunow	r		-	-	-	-	-	-
Nitzschia dissipata (Kützing) Grunow	f	f	ak	ot	o-α	meso	3	
Nitzschia fonticola (Grunow) Grunow	f	f	ak	oe	o-β	oligo-meso	1	
Nitzschia inconspicua Grunow	r	r	ak	ot-h	β-α	meso-eu	3	**
Nitzschia linearis (C. Agardh) W. Smith	r	r	ak	ot-h	β-α	meso-eu	3	
Nitzschia palea (Kützing) W. Smith		r	n	ot	o-α	meso-eu	3	
Nitzschia perminuta (Grunow) M. Peragallo		r	ak	h	o	oligo-meso	3	*
Nitzschia recta Hantzsch ex Rabenhorst		r	ak	ot	β	meso	1	
Nitzschia sociabilis Hustedt	r	r	n	ot-h	β-α	meso-eu	1	**
Nitzschia solgensis Cleve-Euler		r	ak	oe-ot	o-β	oligo-meso	4	V
Odontidium hyemale (Roth) Kützing	r	r	ak	hb	o	hypo	2	*
Odontium mesodon (Kützing) Kützing	r	a	n	hb-oe	o	oligo	2	*
Pinnularia dubitabilis Hustedt	r		-	h	-	-	-	-
Pinnularia sp.		r						
Planothidium frequentissimum (Lange-Bertalot) Lange-Bertalot	f	f	ak	hb-oe	α-poli	ind	-	**
Planothidium lanceolatum (Brébisson ex Kützing) Lange-Bertalot	a	a	ak	hb-oe	o	oligo	3	
Platessa hustedtii (Krasske) Lange-Bertalot	r	r	ak	hb-oe	o	oligo	4	*
Reimeria sinuata (W. Gregory) Kociolek & Stoermer	r		n	oe	o	oligo	3	**
Reimeria uniseriata S.E. Sala, J.M. Guerrero & Ferrario	r		-	-	-	-	-	-
Rhoicosphenia abbreviata (C. Agardh) Lange-Bertalot	r	r	ak	oe	o-β	oligo-meso	-	**
Sellaphora sp.	r		-	-	-	-		-
Simonsenia delognei (Grunow) Lange-Bertalot	r	r	-	ot	α	eu	3	**
Ulnaria biceps (Kützing) Compère		r	ak	h	-	eu	-	G
Ulnaria ulna (Nitzsch) Compère		r	ak	ot	β	meso	2	

10. Chapter VI

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Diatom assemblages from different substrates of the Casteldoria thermo-mineral spring (Northern Sardinia, Italy)

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ABSTRACT

The thermo-mineral springs of Sardinia have historically been the object of several geochemical studies, whereas the biota and ecology have been totally neglected. The aim of this work is to contribute to the knowledge of the diatom microflora and ecology of the Casteldoria spring (northern Sardinia), in the hottest geothermal district of the Island. A total of 132 diatom taxa (56 genera) were found from three investigated substrates: rock (near the water emergence point), cobbles and sediments (in the spring-fed rivulet). Overall, 62 taxa (47% of the total) were common to other thermal springs of the Mediterranean area. Significant differences in the species composition among all substrates were highlighted by the ANOSIM test (global $R = 0.554$; $p = 0.1\%$). The assemblages from rock formed a separated cluster from assemblages from cobbles and sediments in the nMDS plot. Rock-dwelling assemblages also had a lower species richness, probably due to the higher water temperature. Total suspended solids, discharge, water temperature and bromine were the most correlated environmental variables with diatom species and seem to influence both species composition and structure of assemblages. Some abundant taxa, such as *Pinnularia joculata* and *Rhopalodia operculata*, which fit well on high temperature and mineral content, can be considered representative taxa of the studied spring. This study underlines the importance of investigating different substrates to document diatom biodiversity in a more exhaustive way. It contributes to the knowledge of diatoms and ecology in thermo-mineral springs of Sardinia, providing first information on species composition at small-spatial and seasonal scale.

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Introduction

Thermo-mineral springs are characterized by temperature values and electrolyte contents exceeding those of other types of springs (Spahić and Temimović 2014; Stavreva-Veselinovska and Todorovska 2010). Their waters are well known and have been used for curative and recreational purposes since ancient times (Glazier 2014). Thermo-mineral springs have great socio-economic importance and the interest in this resource has increased over the last decades, mainly because of diversification in water uses (Olivier, Venter, and Jonker 2011). In fact, they are exploited in industrial processing, heating settlements, agriculture and greenhouses, aquaculture, bottle water and the extraction of rare elements (Bahati 2003; Baradács et al. 2001; Lund 2000; Lund and Freeston 2001; Shevenell et al. 2002; Vimmerstedt 1998). In several countries of the world, thermo-mineral waters are increasingly used in health and well-being sector (Smith and Puczkò 2009) and power generation (Lund, Freeston, and Boyd 2005; Petraccia et al. 2006), contributing to the control of greenhouse gas emissions of anthropogenic origin (Holm, Jennejohn, and Blodgett 2012).

The particular conditions that may occur in thermo-mineral springs – such as high temperature, low pH, the presence of hydrogen sulphide – make them interesting places to study specific algal assemblages (DeNicola 2000; Hambrook, Armitage, and Vis 1999; Quintela et al. 2013). Diatoms are a group of microalgae able to survive in various ecological niches (Nikulina and Kociolek 2011) and also occur in extreme environments, including hot springs (Bourrelly and Manguin 1946; Hobbs et al. 2009; Mannino 2007; Owen, Renaut, and Jones 2008; Yoshitake, Fukushima, and Lepskaya 2008). Diatoms can be used as a useful tool in the planning of sustainable use and preservation of water resources (Mogna et al. 2015), because they are very sensitive to environmental variables and their ecological responses are well understood (e.g. Prygiel and Coste 1993). Studies on diatoms from hot springs have been carried out in many geographic

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Figure 1. Geographic location of the Casteldoria thermo-mineral spring.

areas but overall their number remains limited (Nikulina and Kociolek 2011).

In Sardinia, the thermo-mineral springs have hystorically been the object of several geochemical studies (e.g. Caboi et al. 1985, 1988; D'Amore, Fancelli, and Caboi 1987; Frau 1994; Nuti et al. 1977). Conversely, their biota and ecology have been totally neglected so far. Quantitative knowledge of this kind of spring in the region is also incomplete and the resource is still largely unused (Brandis 1973; RAS 2013).

The main objectives of this work were: (1) to document the diatom species composition of the Casteldoria thermo-mineral spring using optical (LM) and scanning electron microscopy (SEM); (2) to test differences between assemblages collected from three different substrates (rock, cobbles, fine sediments); (3) to explore the relationships between diatom taxa and environmental variables.

Material and methods

Study site

The thermal area of Casteldoria is located in northern Sardinia, in the region of Anglona, the hottest geothermal district of the Island (Figure 1). The thermal circuit is fed by meteoric water infiltrating the granitic massif of Tempio Pausania at a mean altitude of c. 550 m a.s.l (Cuccuru, Oggiano, and Funedda 2015). The thermo-mineral waters emerge from different springs, and their presence is revealed by gas emissions both along the banks and on the surface of the Coghinas River. The selected spring (40°54'019" N 008°53'951"E) is one of the most interesting at the regional level because of its high temperature (up to 75°C) and salinity (5 g L^{-1}) (Minissale et al. 1999). The water emerges from granites at an elevation of about 6 m a.s.l., at the intersection between two normal oblique faults (Cuccuru, Oggiano, and Funedda 2015; Sacchi et al. 2008). The spring is a rheocrene feeding a rivulet that flows into the Coghinas River a short distance from the emergence point. Water chemistry was classified as Na–Cl–Ca, with N_2 gas emissions and appreciable amounts of radon and helium (e.g. Cuccuru, Oggiano, and Funedda 2015; Minissale et al. 1999; Nuti et al. 1977). The discharge is very low (<0.5 L s^{-1}) due to strong water abstraction by a nearby thermal medicine rehabilitation centre.

Sampling

Water samples for physical and chemical analyses were collected monthly, from May 2016 to April 2017, using 1-L polyethylene bottles. The samples for dissolved-oxygen were collected in 150 mL glass bottles, and immediately fixed *in situ* for the laboratory analyses with the Winkler method (APAT/IRSA–CNR 2003).

The sampling of benthic diatoms was carried out on a complete seasonal cycle (spring: May and June; summer: July and September; autumn: October and December; winter: January and April). The samples were collected from three different substrates (rock, cobbles, fine sediments) following the methods reported in Kelly et al. (1998) and ISPRA (2014). Epilithic diatoms were collected by scraping the rock surface close to the water emergence point and the upper surface of hard natural substrates (five cobbles randomly selected along the spring-fed rivulet) with a hard-bristled toothbrush. Epipelic diatoms were collected from fine surface sediments along the spring-fed rivulet using glass tubes. All samples were preserved in 100 mL polyethylene bottles and fixed *in situ* with a formaldehyde solution (4% v/v).

Measurements and analyses

Water temperature (T° C) was measured *in situ* at three different points of the spring using a digital thermometer (Temp 7 RTD basic with immersion probe PT100). Repeated measurements of discharge (Q) were made by the volumetric method using a 1 L bottle and a chronometer. pH, conductivity (Cond), dissolved oxygen (DO), alkalinity (Alk), chlorides (Cl^-), total hardness (THar), biological oxygen demand (BOD_5), total suspended solids (TSS), Ca^{2+}, Mg^{2+}, Fe^{2+} and Mn^{2+}, soluble reactive phosphorus ($P\text{-}PO_4^{3-}$), total phosphorus (TP), ammonia nitrogen ($N\text{-}NH_4^+$), nitrites ($N\text{-}NO_2^-$), nitrates ($N\text{-}NO_3^-$), total nitrogen (TN), and reactive silica (RSi) were measured in the laboratory using standard methods reported by APAT/IRSA–CNR (2003), APHA (1998) and Strickland and Parsons (1972). Other ions and trace elements, such as bicarbonate (HCO_3^-), carbonate (CO_3^{2-}), iodine (I^-), fluorine (F^-), bromine (Br^-), potassium (K^+), sodium (Na^+) and sulfate (SO_4^{2-}), were measured in the same months of the diatom sampling at EN.A.S (Ente Acque della Sardegna) laboratory, with the methods reported by APAT/IRSA–CNR (2003).

Diatom subsamples (50 mL) were treated in the laboratory, after natural decantation for 48 h. The organic content of frustules was eliminated by an oxidation process on a heating plate with hydrogen peroxide (30% v/v). After some washings with distilled water, a small amount of homogeneous sample (about 100 μL) was placed on microscope slides using Styrax resin (refractive index = 1.59).

Diatom taxa observations and counts were performed using a light microscope (LM, Zeiss Axiovert 10, equipped with phase–contrast and micrometric scale) at 1000× magnification. LM images were taken with a Leica DC500 camera mounted on a Leica DMRX microscope. For SEM analysis subsamples of the diatom suspension were air-dried on aluminium sheets and fixed on aluminium stubs that were sputter-coated with gold (Sputter Coater Edwards S-150A). A part of the subsamples, mounted on aluminium stubs and coated with platinum using a Modular High Vacuum Coating System BAL-TEC MED 020 (BAL-TEC AG, Balzers, Liechtenstein), was also analysed by an ultrahigh-resolution analytical field emission (FE) scanning electron microscope, Hitachi SU-70 (Hitachi High-Technologies Corporation, Japan).

Diatom species were identified using Krammer and Lange-Bertalot (1986, 1988, 1991a, 1991b, 2000), Krammer (2000), Lange-Bertalot et al. (2003), Werum and Lange-Bertalot (2004), Taylor, Harding, and Archibald (2007), Levkov (2009), Żelazna-Wieczorek (2011), and related literature (e.g., Cejudo-Figueiras et al. 2011; Delgado et al. 2015; Gibson, Anderson, and Haworth 2003; Taylor et al. 2014; Tofilovska et al. 2014; Tuji and Houki 2004; Tuji and Williams 2006; Wetzel et al. 2015).

Data and statistical analyses

Diatom count data were converted into percentage relative abundances for the statistical analyses. A non-metric multidimensional scaling ordination technique (nMDS) was made to explore the similarity between assemblages from three different substrates (rock, cobbles and sediments) (software Primer 5). A Bray-Curtis similarity matrix was constructed using log (x + 1) abundance data from all species with a relative abundance ≥1.5% in at least one sample. The significance of the differences was validated by a one-way analysis of similarities (ANOSIM). ANOSIM generates a value of the R statistic representing the strength of the factors on the samples. A R value close to 1 indicates high separation between factor levels, a value of 0 represents the null hypothesis indicating no difference. For this analysis, probability percentages (p) <0.03 were considered significant. The ANOSIM pairwise test was also performed to test each pair of substrates. To support ANOSIM, the percentage level of similarity between diatom assemblages from the investigated substrates and the percentage contribution of each species to the differences were determined by the SIMPER analysis. The species are classified from the highest to the lowest contribution. A cut-off criterion was applied to allow identification of a subset of species whose cumulative percentage contribution reached 70% of the dissimilarity value. Spearman's rank correlation analyses were used to test relationships between diatoms (all taxa with relative abundance ≥1.5% in at least one sample) and 15 selected environmental variables: T° C, Q, pH, Cond, BOD_5, Cl^-, TP, N-NO_3^-, Rsi, TSS, Br^-, Ca^{2+}, Na^+, SO_4^{2-}, and Fe^{2+} (software R version 3.4.1). The assumptions of the Spearman correlation are that data must be at least ordinal and the scores on one variable must be monotonically related to the other variable. Values of the correlation coefficient (p) determining the strength of the relationship are given. Correlation coefficients >0.50 represent a large association or relationship. The significance of observed values of correlation coefficients were tested by mean of the Student's t distribution with $n - 2$ degrees of freedom. Probability percentages (p) <0.05 were considered significant.

Results

Environmental variables

Results concerning physical and chemical variables are reported in Table 1. The water temperature mean annual value was 70.7 °C at the emergence point and 59°C in the spring-fed rivulet. Values of pH were almost close to neutrality, only slightly acid (6.3–6.9) in a few months. The water oxygenation had values ranging from 0.7 to 5.1 mg L^{-1} and percentage saturation from 15 to 118%. The conductivity values ranged between 8120 and 9180 μS cm^{-1}. The range of values for Cl^- and SO_4^{2-} was respectively 2530–3701 mg L^{-1} and 88.7–100.8 mg L^{-1}. Total hardness showed values ranging from 1013 to 1600 mg $CaCO_3$ L^{-1}. Nutrient contents, as annual mean values, were 108 μg L^{-1} for N-NO_3^- and 23 μg L^{-1} for P-PO_4^{3-}. BOD_5 values ranged from 0 to 1.6 mg L^{-1}. Na^+, Cl^- and Ca^{2+} ions showed mean annual values respectively of 1242 mg L^{-1}, 2883 mg L^{-1} and 54.8 mg L^{-1}. The annual mean values of Fe^{2+} (0.05 mg L^{-1}) and Mn^{2+} (0.14 mg L^{-1}) were lower than the critical limits for aquatic life, respectively of 1 and 1.09 mg L^{-1}, indicated by the United States Environmental Protection Agency guidelines (Stephen et al. 1985).

Table 1. Range, mean and standard deviation values of the physical and chemical variables recorded and measured in the Casteldoria spring ($n = 12$). D.L. = Detection Level.

Variables	Min–Max	Mean ± St. Dev.
Temperature at the emergence point (°C)	67.8–71.7	70.7 ± 1.2
Temperature at the spring-fed rivulet (°C)	54.5–63.8	59 ± 2.2
Discharge ($L\ s^{-1}$)	0.009–0.038	0.022 ± 0.009
pH (units)	6.3–7.3	7 ± 0.4
Alkalinity (meq L^{-1})	0.25–0.60	0.47 ± 0.10
Conductivity ($\mu S\ cm^{-1}$ at 25°C)	8120–9180	8685 ± 338
Dissolved Oxygen (mg L^{-1})	0.7–5.1	3.2 ± 1.5
Oxygen (% saturation)	15–118	74.5 ± 34.9
BOD_5 (mg O_2 L^{-1})	0–1.6	1.4 ± 1.4
Cl^- (mg L^{-1})	2530–3701	2883 ± 266.0
Total hardness (mg $CaCO_3$ L^{-1})	1013–1600	1447 ± 149
Reactive phosphorus (µg P L^{-1})	10–47	23 ± 11
Total phosphorus (µg P L^{-1})	18–187	94 ± 55
N-NO_2^- (µg N L^{-1})	1–6	2.5 ± 2.0
N-NO_3^- (µg N L^{-1})	23–179	108 ± 45
N-NH_4^+ (µg N L^{-1})	123–325	244 ± 54
Total nitrogen (µg N L^{-1})	536–559	977 ± 342
Reactive silica (mg L^{-1})	25.2–29.9	27.5 ± 1.6
Total Suspended Solids (mg L^{-1})	6.2–46.0	18.4 ± 11.7
HCO_3^- (mg L^{-1})	25.6–30.5	27.9 ± 2.0
CO_3^{2-} (mg L^{-1})	<D.L.	<D.L.
I^- (mg L^{-1})	<D.L.	<D.L.
F^- (mg L^{-1})	0.4–3.4	1.8 ± 0.9
Br^- (mg L^{-1})	<D.L.–9.8	7.8 ± 3.0
K^+ (mg L^{-1})	36.7–42.9	41.6 ± 2.0
Mg^{2+} (mg L^{-1})	222–355	320 ± 35
Ca^{2+} (mg L^{-1})	40–60	54.8 ± 5.3
Na^+ (mg L^{-1})	1200 –1439	1242 ± 80
SO_4^{2-} (mg L^{-1})	88.7–100.8	93.5 ± 5.0
Fe^{2+} (mg L^{-1})	0.03–0.08	0.05 ± 0.02
Mn^{2+} (mg L^{-1})	0.04–0.22	0.14 ± 0.07

Diatom assemblages

The list of the diatom species recorded in each substrate with their class of relative abundance (RA) is presented in Table 2. A total of 132 diatom taxa from 56 genera were found, of which 13 (8 genera) were centric and 119 (48 genera) pennate. Species richness ranged from 73 (38 genera) in the rock to 109 (51 genera) in the cobbles. The most species-rich genera were *Cocconeis* and *Halamphora* with a maximum respectively of 5 and 6 taxa in the sediments, *Gomphonema* with a maximum of 7 taxa and *Navicula* and *Nitzschia* with a maximum of 14 and 10 taxa in the cobbles. Overall, 3 taxa from 3 genera occurred only in the samples from rock, 20 taxa from 17 genera only in the samples from cobbles, and 13 taxa from 13 genera only in the samples from sediments. The number of species common to samples collected from all substrates was 58 from 34 genera (44% of the total species). Among these, *Aulacoseira granulata*, *A. ambigua*, *A. subarctica* [including *A. pusilla* (F. Meister) Tuji & Houki], *Halamphora minutissima*, *Nitzschia* sp., *Pinnularia joculata*, and *Stephanodiscus neoastraea* were abundant in all substrates (RA>5%). Other taxa, such as *Nitzschia microcephala*, *N. perspicua* and *Rhopalodia operculata* were abundant (RA>5%) in at least one substrate. The taxa frequent on at least one substrate (RA 1.5–5%) were *Achnanthidium exiguum*, *Diadesmis confervacea*, *Luticola goeppertiana*, *Nitzschia clausii*, and *N.* aff. *subrostrata*. Among the rare taxa (RA ≤ 1.5% in at least one substrate), we observed for example *Achnanthes coarctata*, *Nitzschia filiformis*, and *N. linearis*. Some of these abundant, frequent and rare taxa and some diatoms observed in the samples but not present in the counts, are reported with LM (Figures 2–41) and SEM (Figures 42–76) images. Teratological forms with deformities in the valve outline were observed for *A. coarctata*, *Cocconeis euglypta*, *Fragilaria crotonensis*, *Neidiomorpha binodis*, *Sellaphora seminulum*, *Nitzschia* sp. and *Ulnaria ulna* in the assemblages from cobbles (RA 0–0.4%), and for *Navicula tripunctata*, *N. binodis*, and *U. ulna* in the assemblages from sediments (RA 0–0.2%).

The species composition based on the five most abundant taxa for each substrate in all seasons and within each season are presented in Figures 77 and 78. Considering each substrate in all seasons (Figure 77), larger variations in the relative abundance were observed for *R. operculata* and *Nitzschia* sp. in the rock, *H. minutissima* and *A. ambigua* in the cobbles, and *Nitzschia* sp. and *S. neoastraea* in the sediments. Considering the three substrates within each season (Figure 78), larger variations in the relative abundance were observed for *R. operculata* and *A. granulata* in spring, *A. ambigua* and *A. granulata* in summer, *P. joculata* and *Nitzschia* sp. in autumn, *H. minutissima* and *P. joculata* in winter. Further, differences were observed in the presence/absence of species, excluding *A. granulata*, *Nitzschia* sp. and *S. neoastraea* present in all substrates.

The species composition based on the centric diatoms present for each type of substrate in all seasons is presented in Figure 79. The rock was the substrate with the lowest percentage abundance of centric forms in all seasons. The highest percentage abundances were observed in summer on cobbles and in winter on sediments.

The nMDS ordination (Figure 80) mainly showed a clear separation between assemblages from rock and assemblages from cobbles and sediments. The ANOSIM test (global $R = 0.554$; $p = 0.1\%$) revealed significant differences in the assemblages from the three substrates, also highlighted by the pairwise tests (Table 3).

According to the Simper analysis (Table 4) the highest dissimilarity levels were found between assemblages from rock and cobbles (44.60%) and assemblages from rock and sediments (39.59%). In both cases, the greatest contribution to the differences was provided by *P. joculata* and *R. operculata*. The lowest dissimilarity level (32.67%) was found between assemblages from cobbles and sediments. The greatest contribution to the differences was provided by *N. microcephala* and *H. minutissima*.

The Spearman tests (Tables 5, 6 and 7) revealed 48 significant correlations between diatom taxa and environmental variables in all substrates, of which 9 were most significant ($p<0.01$) and 39 significant ($p<0.05$). Total suspended solids, discharge, water temperature and bromine were respectively the environmental variables with the largest number of correlations with diatom species.

Table 2. Species composition of diatom assemblages from three different substrates (rock, cobbles and sediments).

	Epilithon		Epipelon
Taxon name	rock	cobbles	sediments
**Achnanthes coarctata* (Brébisson ex W. Smith) Grunow	1	1	1
**Achnanthidium exiguum* (Grunow) Czarnecki	1	1	2
**Achnanthidium minutissimum* (Kützing) Czarnecki	1	1	1
Achnanthidium subatomus (Hustedt) Lange-Bertalot	1	1	2
Amphora copulata (Kützing) Schoeman & R.E.M.Archibald	0	1	1
**Amphora ovalis* (Kützing) Kützing	1	1	0
**Amphora pediculus* (Kützing) Grunow	1	1	1
Aulacoseira ambigua (Grunow) Simonsen	3	3	3
**Aulacoseira granulata* (Ehrenberg) Simonsen	3	3	3
Aulacoseira granulata var. *angustissima* (O. Müller) Simonsen	1	1	1
Aulacoseira subarctica (O. Müller) E.Y.Haworth	3	3	3
**Bacillaria paxillifera* (O.F. Müller) T.Marsson	1	1	1
Caloneis amphisbaena (Bory) Cleve	0	0	1
**Caloneis lancettula* (P. Schulz) Lange-Bertalot & Witkowski	0	1	0
**Cocconeis euglypta* Ehrenberg	2	1	2
**Cocconeis pediculus* Ehrenberg	1	2	2
**Cocconeis placentula* Ehrenberg	1	1	1
**Cocconeis placentula* var. *lineata* (Ehrenberg) Van Heurck	1	0	1
Cocconeis pseudolineata (Geitler) Lange-Bertalot	1	1	1
**Cosmioneis pusilla* (W. Smith) D.G. Mann & A.J.Stickle	1	1	0
Craticula subminuscula (Manguin) C.E.Wetzel & Ector	0	1	0
Cyclostephanos dubius (Hustedt) Round	2	2	2
**Cyclotella meneghiniana* Kützing	2	2	2
**Cymbella tumida* (Brébisson) Van Heurck	0	1	0
Denticula subtilis Grunow	1	1	1
**Denticula tenuis* Kützing	0	0	1
**Diadesmis confervacea* Kützing	1	2	2
Diatoma mesodon (Ehrenberg) Kützing	0	0	1
**Diatoma vulgaris* Bory	1	1	1
Diploneis cf. *elliptica* (Kützing) Cleve	1	0	0
Diploneis sp.	0	1	1
Discostella stelligera (Cleve & Grunow) Houk & Klee	0	0	1
**Ellerbeckia arenaria* (Moore ex Ralfs) R.M.Crawford	0	1	1
**Encyonema leibleinii* (C. Agardh) W.J.Silva, R.Jahn, T.A.V.Ludwig & M. Menezes	0	1	1
**Encyonema ventricosum* (C. Agardh) Grunow	1	1	1
Epithemia adnata (Kützing) Brébisson	0	1	1
**Epithemia turgida* (Ehrenberg) Kützing	0	1	1
Eunotia minor (Kützing) Grunow	0	1	1
Eunotia pectinalis (Kützing) Rabenhorst	1	0	1
**Fallacia pygmaea* (Kützing) Stickle & D.G.Mann	0	0	1
**Fragilaria candidagilae* Almeida, C.Delgado, Novais & S.Blanco	0	0	1
Fragilaria crotonensis Kitton	1	1	1
**Fragilaria vaucheriae* (Kützing) J.B.Petersen	0	1	1
**Frustulia vulgaris* (Thwaites) De Toni	0	1	0
**Gomphonema* aff. *gracile* Ehrenberg	0	1	0
Gomphonema italicum Kützing	0	1	0
**Gomphonema minutum* (C.Agardh) C.Agardh	1	1	1
**Gomphonema olivaceum* (Hornemann) Brébisson	0	1	1
**Gomphonema parvulum* Kützing	0	1	1
Gomphonema rhombicum M.Schmidt	0	1	0
Gomphonema sp. 1	0	1	0
Gomphonema sp. 2	0	0	1
Gyrosigma acuminatum (Kützing) Rabenhorst	0	0	1
Halamphora minutissima (Kützing) C.E.Wetzel & Compère	3	3	3
Halamphora cf. *normanii* (Rabenhorst) Levkov	0	1	1
Halamphora paraveneta (Lange-Bertalot, Cavacini, Tagliaventi & Alfinito) Levkov	0	1	1
Halamphora sp.	1	1	1
**Halamphora veneta* (Kützing) Levkov	1	1	1
**Hantzschia amphyoxis* (Ehrenberg) Grunow	1	1	1
Hippodonta capitata (Ehrenberg) Lange-Bertalot, Metzeltin & Witkowski	0	1	1
**Hippodonta hungarica* (Grunow) Lange-Bertalot, Metzeltin & Witkowski	1	1	1
Humidophila contenta (Grunow) R.L. Lowe, Kociolek, J.R.Johansen *et al.*	0	1	1
Karayevia clevei (Grunow) Round	1	1	1
Kolbesia gessneri (Hustedt) Aboal	0	1	0
**Lemnicola hungarica* (Grunow) Round & Basson	1	1	1
Luticola acidoclinata Lange-Bertalot	1	0	0
Luticola goeppertiana (Bleisch) D.G.Mann ex Rarick et al.	1	2	1
**Luticola mutica* (Kützing) D.G.Mann	1	1	1
**Luticola nivalis* (Ehrenberg) D.G.Mann	1	0	1
Luticola sp.	2	1	1
Luticola ventriconfusa Lange-Bertalot	0	1	0
**Melosira varians* C.Agardh	1	1	1
Navicula antonii Lange-Bertalot	1	0	0
Navicula capitatoradiata H.Germain	1	1	1
**Navicula cincta* (Ehrenberg) Ralfs	0	1	1

(*Continued*)

Table 2. (*Continued*).

	Epilithon		Epipelon
Taxon name	rock	cobbles	sediments
**Navicula cryptocephala* Kützing	2	1	0
**Navicula erifuga* Lange-Bertalot	2	2	2
**Navicula gregaria* Donkin	2	2	2
Navicula lanceolata Ehrenberg	0	1	1
Navicula radiosa Kützing	1	1	0
Navicula recens (Lange-Bertalot) Lange-Bertalot	1	1	2
Navicula rhyncocephala Kützing	1	0	1
Navicula rostellata Kützing	0	1	0
Navicula striolata (Grunow) Lange-Bertalot	0	1	0
Navicula symmetrica R.M.Patrick	0	1	1
Navicula tenelloides Hustedt	0	1	1
**Navicula tripunctata* (O.F.Müller) Bory	1	1	1
**Navicula veneta* Kützing	1	2	0
**Neidiomorpha binodis* (Ehrenberg) Cantonati, Lange-Bertalot & Angeli	2	1	1
**Nitzschia amphibia* Grunow	1	2	2
**Nitzschia clausii* Hantzsch	1	2	2
**Nitzschia dissipata* (Kützing) Rabenhorst	0	1	0
**Nitzschia filiformis* (W.Smith) Van Heurck	1	1	1
**Nitzschia fonticola* (Grunow) Grunow	0	2	1
Nitzschia microcephala Grunow in Cleve & Möller	3	2	3
**Nitzschia palea* (Kützing) W.Smith	3	2	2
Nitzschia perspicua Cholnoky	2	1	3
Nitzschia recta Hantzsch ex Rabenhorst	0	0	1
Nitzschia sp.	3	3	3
Nitzschia aff. *subrostrata* Hustedt	2	2	2
Nitzschia umbonata (Ehrenberg) Lange-Bertalot	1	0	1
**Nitzschia vitrea* G. Norman	1	0	1
Opephora olsenii Møller	0	1	0
Pinnularia joculata (Manguin) Krammer	3	3	3
Pinnularia sp.	0	1	0
Placoneis sp.	0	1	0
Planothidium frequentissimum (Lange-Bertalot) Lange-Bertalot	0	1	1
**Planothidium lanceolatum* (Brébisson ex Kützing) Lange-Bertalot	1	1	1
Platessa hustedtii (Krasske) Lange-Bertalot	0	1	1
Pleurosira laevis (Ehrenberg) Compère	1	1	1
**Pseudostaurosira brevistriata* (Grunow) D.M.Williams & Round	3	1	2
Pseudostaurosira parasitica (W.Smith) E.Morales	0	1	0
Pseudostaurosira subsalina (Hustedt) E.Morales	0	1	1
**Reimeria sinuata* (W. Gregory) Kociolek & Stoermer	0	1	0
**Rhoicosphenia curvata* (Kützing) Grunow	1	1	1
Rhopalodia brebissonii Krammer	1	1	1
**Rhopalodia gibba* (Ehrenberg) Otto Müller	0	0	1
Rhopalodia operculata (C. Agardh) Håkansson	3	2	3
Sellaphora saugerresii (Desmazières) C.E.Wetzel & D.G.Mann	1	2	1
Sellaphora seminulum (Grunow) D.G.Mann	0	1	0
Staurosira venter (Ehrenberg) Cleve & J.D.Möller	0	0	1
Stephanodiscus hantzschii Grunow	0	0	1
Stephanodiscus invisitatus M.H.Hohn & Hellerman	0	1	1
Stephanodicus neoastraea Håkansson & Hickel	3	3	3
Surirella brebissonii var. *kuetzingii* Krammer & Lange-Bertalot	1	1	1
**Surirella ovalis* Brébisson	0	1	0
Tabellaria flocculosa (Roth) Kützing	1	0	1
**Tabularia fasciculata* (C. Agardh) D.M. Williams & Round	0	0	1
**Tryblionella apiculata* W.Gregory	0	1	1
* *Tryblionella levidensis* (W.Smith) Grunow	0	1	0
**Ulnaria monodii* (Guermeur) Cantonati & Lange-Bertalot	0	1	1
**Ulnaria ulna* (Nitzsch) Compère	1	1	1
Number of genera in each substrate	38	51	47
Number of species in each substrate	73	109	103

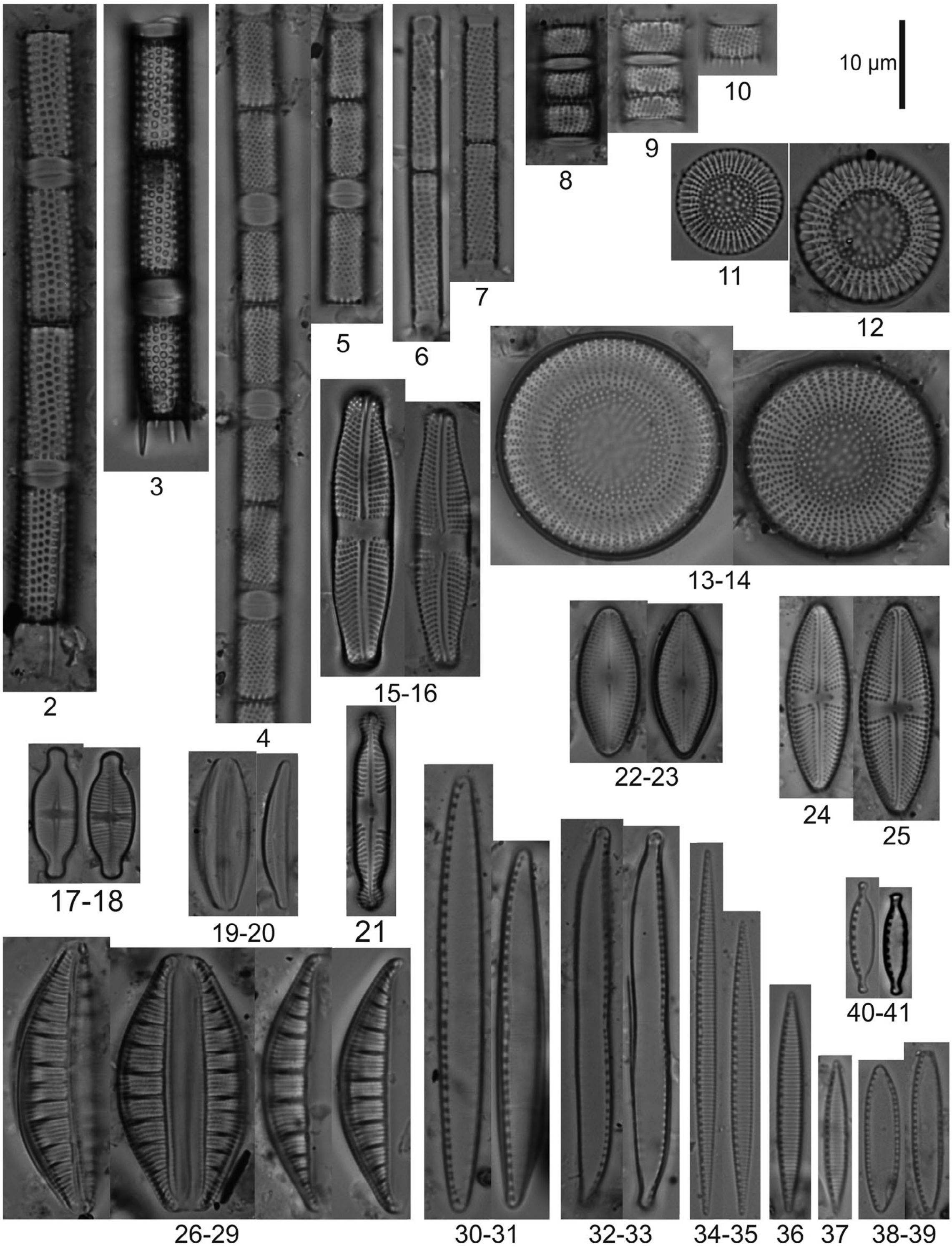
Note: The relative abundance (RA) of species was expressed as follows: 0 = absence, 1 = ≤1.5%, 2 = 1.5–5%, 3 = >5%. * = in common with thermal systems of other Mediterranean areas.

With reference to total suspended solids, the most significant correlations were found for *Cyclostephanos dubius* (positive) and *S. neoastraea* (negative) in the rock and for *Navicula erifuga* (positive) in the sediments. Considering the discharge, the most significant correlations were found for *A. ambigua* (negative) and *P. joculata* (positive) in the cobbles. Finally, temperature and bromine, showed the most significant correlations respectively with *N. erifuga* (positive), and with *A. ambigua* (negative) and *P. joculata* (positive) in the cobbles.

Discussion

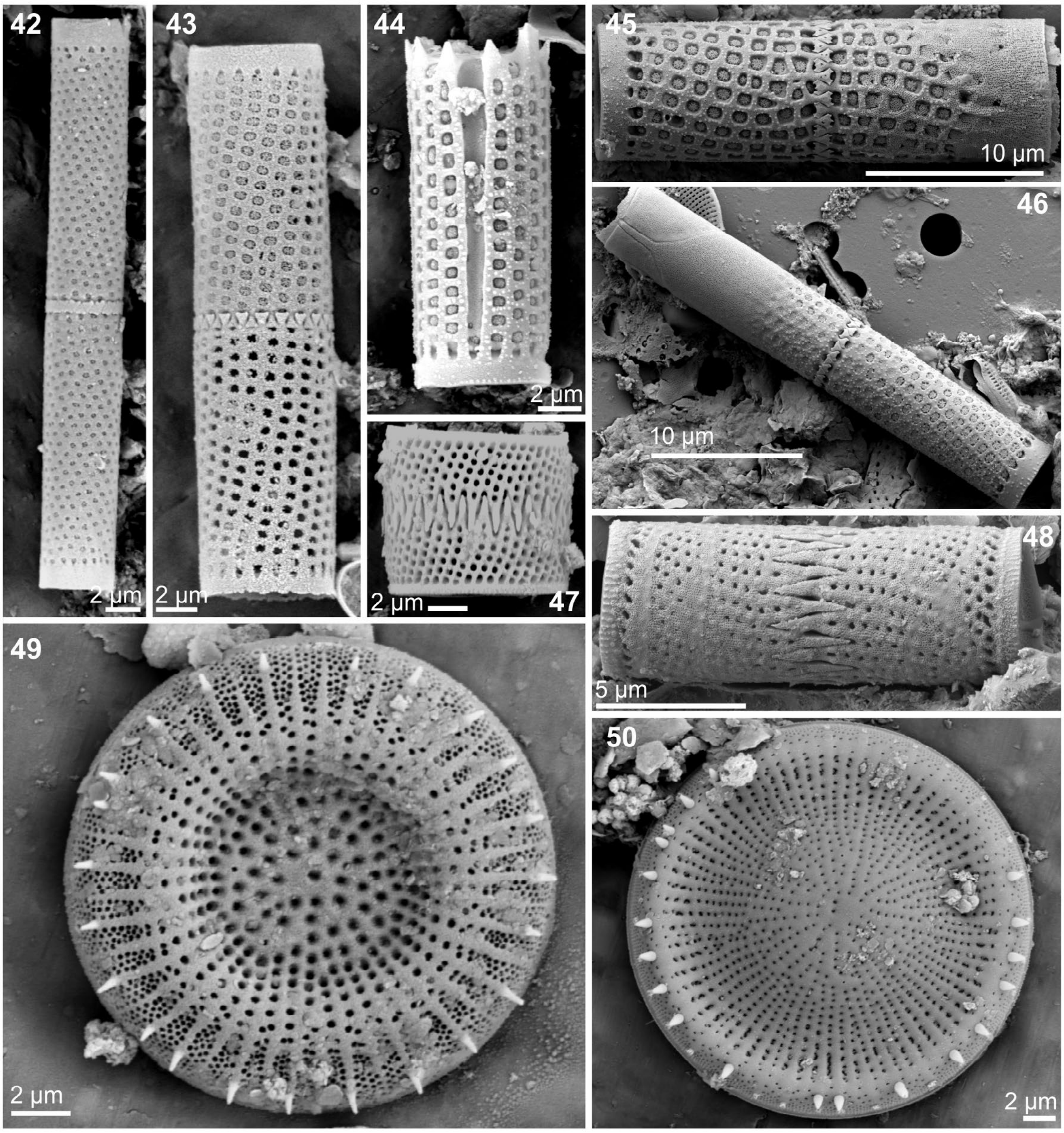
Species richness

The Casteldoria thermo-mineral spring showed a high number of species on all investigated substrates in spite of its small size, the strong water abstraction, high temperature and mineral content. Small size and water abstractions make springs particularly vulnerable to hydromorphological alterations, and may cause an impoverishment of the biota (e.g. Cantonati,



Figures 2–41. Light microscopy (LM). 2–3: *Aulacoseira granulata*; 4–5: *Aulacoseira ambigua*; 6–7: *Aulacoseira granulata* var. *angustissima*; 8–10: *Aulacoseira pusilla*; 11–12: *Cyclostephanos dubius*; 13–14: *Stephanodiscus neoastraea*; 15–16: *Achnanthes coarctata*; 17–18: *Achnanthidium exiguum*; 19–20: *Halamphora minutissima*; 21: *Pinnularia joculata*; 22–23: *Diadesmis confervacea*; 24–25: *Luticola goeppertiana*; 26–29: *Rhopalodia operculata*; 30–31: *Nitzschia filiformis*; 32–33: *Nitzschia clausii*; 34–35: *Nitzschia* aff. *subrostrata*; 36: *Nitzschia* sp.; 37: *Nitzschia* aff. *liebethruthii*; 38–39: *Nitzschia perspicua*; 40–41: *Nitzschia microcephala*.

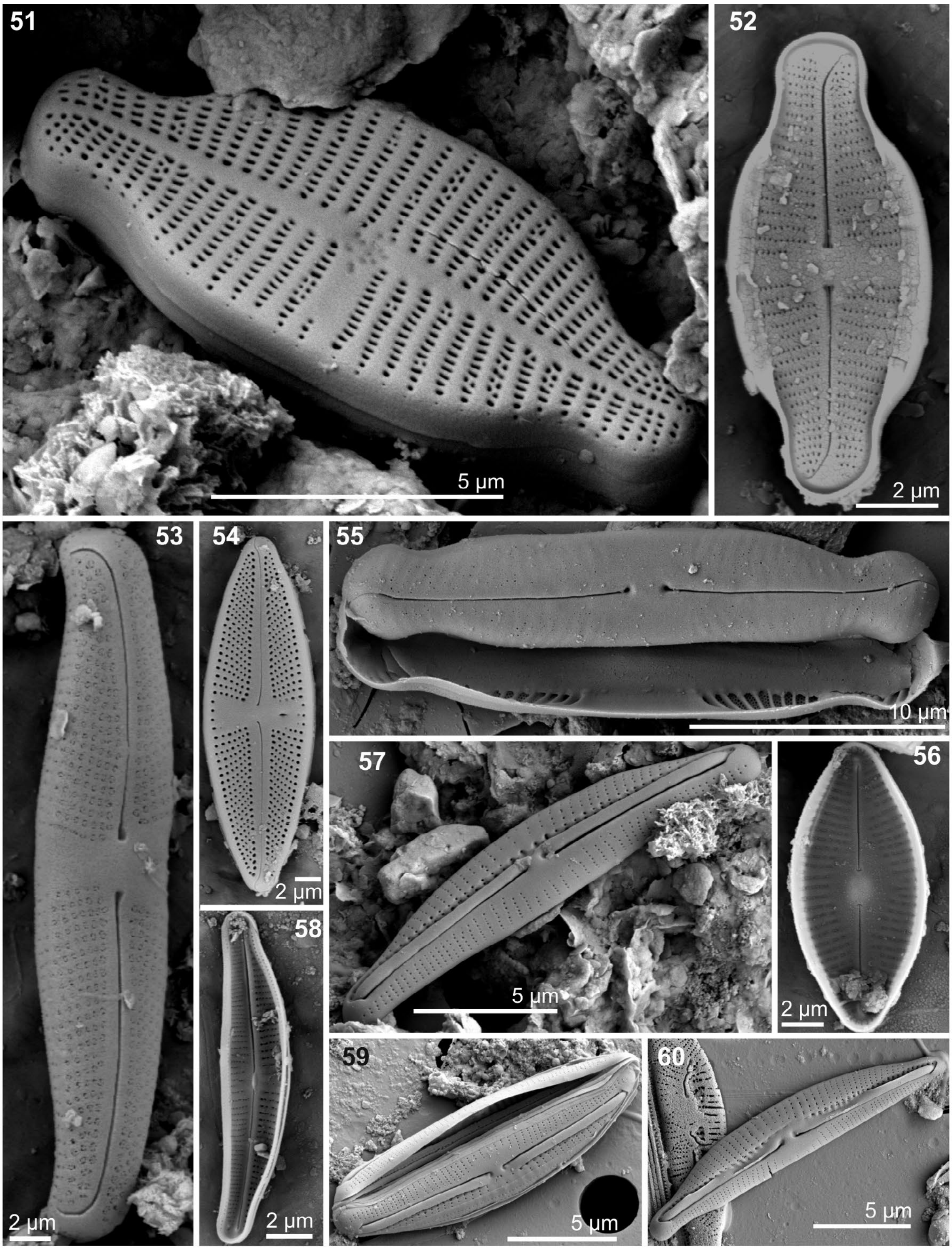
Gerecke, and Bertuzzi 2006; Cantonati et al. 2009, 2012; Scarsbrook, Barquín, and Gray 2007; Weigand 1998). In addition, the high temperature and mineral content in thermal springs affect micro-algal diversity (Jonker, van Ginkel, and Olivier 2013), and these factors are generally considered not very conducive for diatom growth (Mandal and Sarkar 2015). However, diatoms are present in a wide set of ecological niches and also occur in extreme environments (e.g. Hobbs et al. 2009; Malešević et al. 2015; Nikulina and Kociolek 2011), often with taxa specialized for high temperatures (thermophilic species) and high salt content (halophilic species) (Stavreva-Veselinovska and Todorovska 2010). For example, several diatom species were recorded in



Figures 42–50. Scanning electron microscopy (SEM). 42: *Aulacoseira granulata* var. *angustissima*, external mantle view; 43–44: *Aulacoseira granulata*, external mantle view; 45–46: *Aulacoseira ambigua*, external mantle view; 47: *Aulacoseira pusilla*, external mantle view; 48: *Aulacoseira subarctica*, external mantle view; 49: *Cyclostephanos dubius*, external valve view; 50: *Stephanodiscus neoastraea*, external valve view.

thermo-mineral springs from Burundi (29–57.5°C) (Mpawenayo, Cocquyt, and Nindorera 2005), Sicily in Italy (30–38°C) (Mannino 2007), Kuril and Sakhalin Islands in Russia (24–71°C) (Nikulina and Kociolek 2011), Limpopo Province in South Africa (40–45°C) (Jonker, van Ginkel, and Olivier 2013), and São Miguel Island in the Azores (16–80°C) (Quintela et al. 2013). Further, diatoms were found at temperatures of 55°C in Karlovy Vary in the Czech Republic (Kaštovský and Komárek 2001), 76°C at Champagne Pool in New Zealand (Jones, Renaut, and Rosen 1997), 56.8–79.8°C at Puy-de-Dôme, Massif Central in France (Beauger et al. 2017). A higher number of diatom taxa was found in hot springs than in the cold springs of Spain by Leira, Meijide-Failde, and Torres (2017).

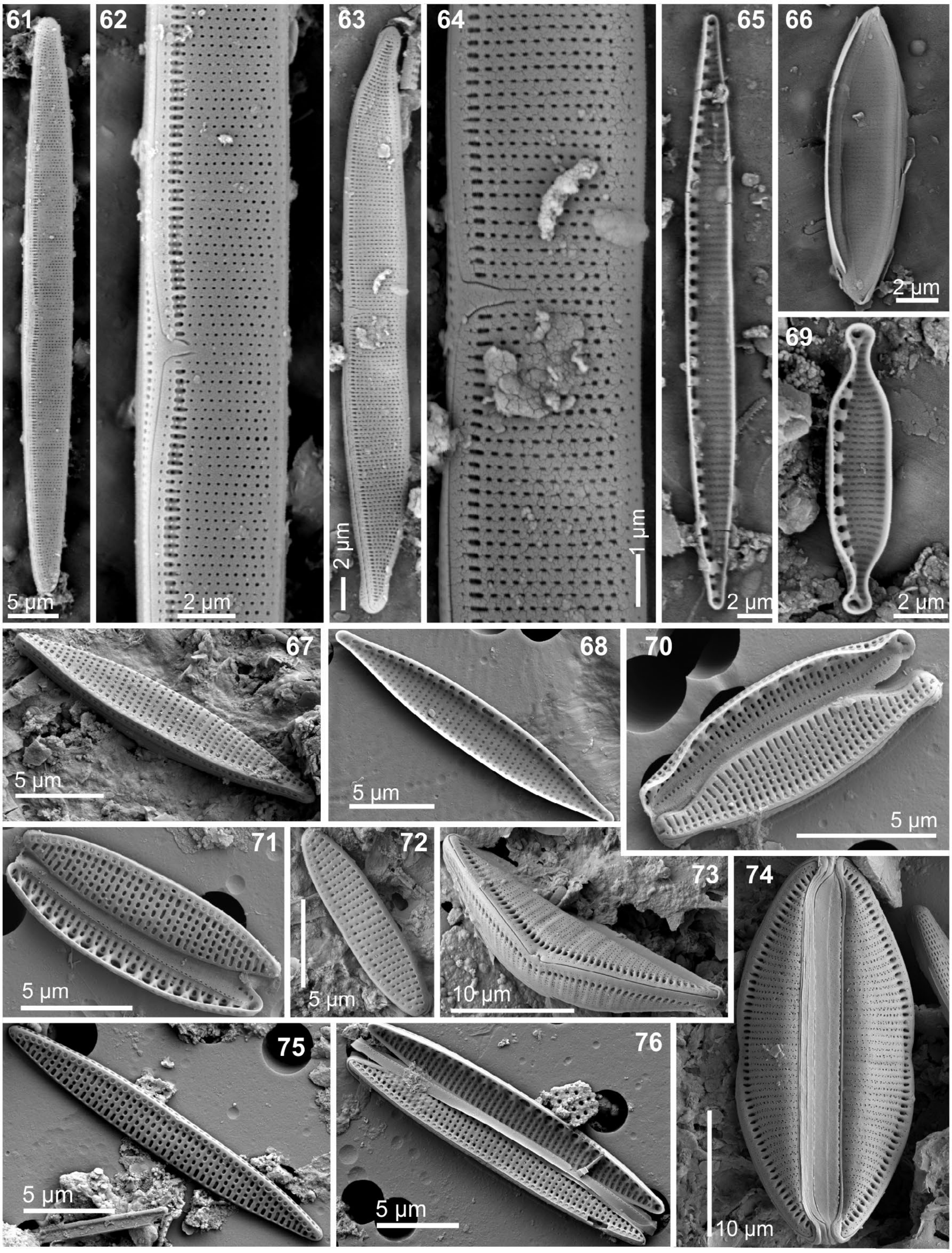
In the Casteldoria spring, the water temperature may be responsible of the lower species richness observed in the assemblages from rock, collected close to the water emergence point. In fact, many studies report a lower diversity of diatoms in warmer waters (e.g. Glazier 2014; Mandal and Sarkar 2015; Schoeman and Archibald 1998; Stockner 1967). The presence of diatoms on this substrate is in agreement with the observations of Jones, Renaut, and Rosen (1997) and Owen, Renaut, and Jones (2008). According to these authors, diatoms can be found in, or close to, high-temperature settings and these substrates may be moistened by steam, water splash, oscillating water levels, or capillary rise, potentially creating cooler and suitable microhabitats.



Figures 51–60. Scanning electron microscopy (SEM). 51–52: *Achnanthidium exiguum*, external rapheless valve view (51), external raphe valve view (52); 53: *Achnanthes coarctata*, external raphe valve view; 54: *Luticola goeppertiana*, external valve view; 55: *Pinnularia joculata*, external valve view and internal valve view; 56: *Diadesmis confervacea*, internal valve view; 57–60: *Halamphora minutissima*, external valve view (57, 60), internal valve view (58), external frustule view (59).

Ecological traits of abundant species and comparison with diatom assemblages from thermal springs of other Mediterranean geographic areas

Despite the high number of species, few diatom taxa (10% of the total) were abundant (RA>5% in at least one sample), suggesting that they are well-adapted to the peculiar environmental conditions found in the spring. They included centric diatoms such as *Aulacoseira ambigua*, *A. granulata*, *A. subarctica*, and *Stephanodicus neoastraea*, in spite of the low water depth due to a strong abstraction. These typically planktonic species are closely linked to the aquatic environment according to Van Dam, Mertens, and Sinkeldam



Figures 61–76. Scanning electron microscopy (SEM). 61–62: *Nitzschia linearis*, external valve view; 63–64: *Nitzschia clausii*, external valve view; 65: *Nitzschia* aff. *subrostrata*, internal valve view; 66: *Nitzschia perspicua*, external valve view; 67–68: *Nitzschia* aff. *liebethruthii*, external valve view (67), internal valve view (68); 69–70: *Nitzschia microcephala*, internal valve view (69), external valve view and internal valve view (70); 71, 75–76: *Nitzschia* sp., external valve view and internal valve view (71, 76), external valve view (75); 72: *Nitzschia* aff. *inconspicua*, external valve view; 73–74: *Rhopalodia operculata*, external valve view (73), external frustule view (74).

(1994). The abundance of these species in the spring is in contrast with the results obtained in thermal rheocrenic springs with shallow waters in Canada, Iceland, and Japan, characterized by a low proportion of centric forms (Villeneuve and Pienitz 1998). However, the presence of these taxa in the spring is consistent with the high mineralization level of water, because they mostly prefer fresh-brackish waters according to Van Dam, Mertens, and Sinkeldam (1994) and Nikulina and Kociolek (2011). In addition, overall, their abundance was higher from September to January, probably due to a greater water availability in the wet rainy period. The

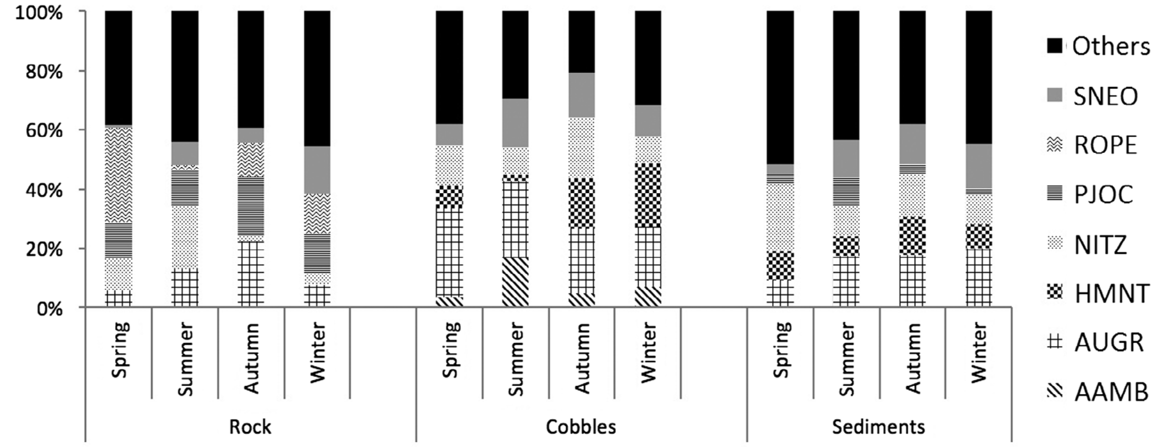


Figure 77. Species composition of diatom assemblages based on the five most abundant species for each substrate. AAMB = *Aulacoseira ambigua*, AUGR = *Aulacoseira granulata*, HMNT = *Halamphora minutissima*, NITZ = *Nitzschia* sp., PJOC = *Pinnularia joculata, ROPE* = *Rhopalodia operculata*, SNEO = *Stephanodiscus neoastraea*.

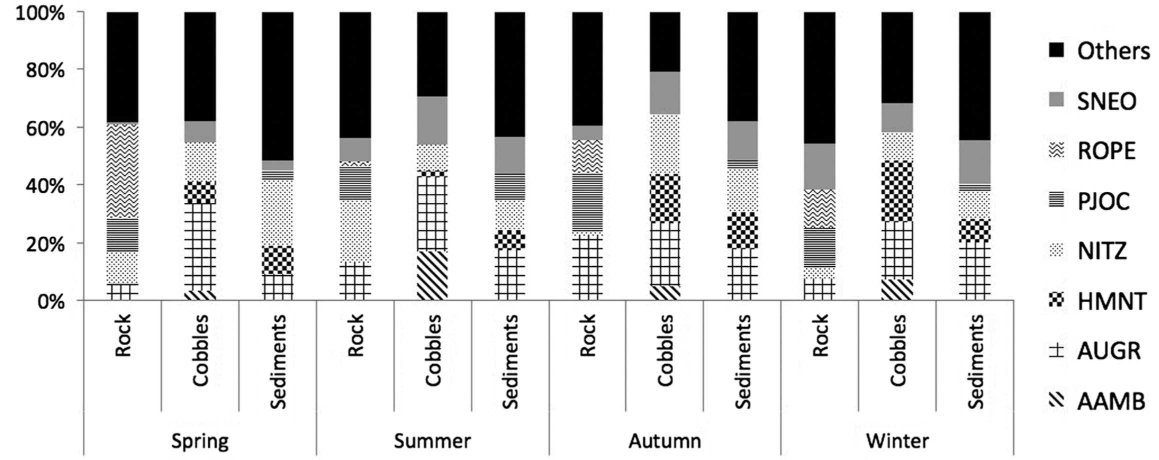


Figure 78. Species composition of diatom assemblages based on the five most abundant species for each season. AAMB = *Aulacoseira ambigua*, AUGR = *Aulacoseira granulata*, HMNT = *Halamphora minutissima*, NITZ = *Nitzschia* sp., PJOC = *Pinnularia joculata*, *ROPE* = *Rhopalodia operculata*, SNEO = *Stephanodiscus neoastraea*.

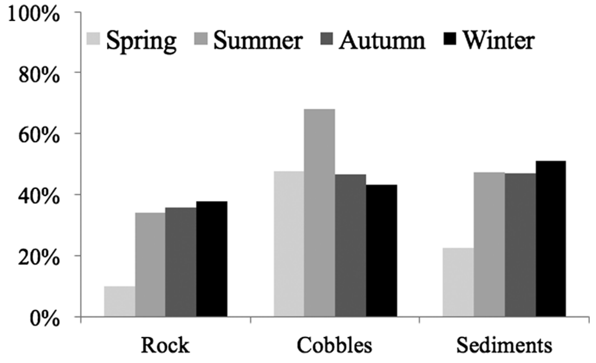


Figure 79. Species composition based on the centric diatoms present for each type of substrate in all seasons. The relative abundance values are expressed as mean values of two samples for each season.

same *Aulacoseira* species were found, although with a low number of specimens, in hot springs from Sakhalin Islands (37–46°C), which are similar in water chemistry to Casteldoria spring but with lower temperature (Nikulina and Kociolek 2011). *Aulacoseira granulata* was also dominant in hot springs associated with Lakes Elmenteita in Kenya (Mpawenayo and Mathooko 2004). Other abundant species in the Casteldoria spring, such as *Navicula recens*, *N. veneta*, *Nitzschia microcephala*, *N. perspicua*, *N. palea*, and *Pseudostaurosira brevistriata*, are generally reported in electrolyte-rich environments and brackish habitats (Coste and Ector 2000; Lange-Bertalot et al. 2017; Taylor, Harding, and Archibald 2007; Van Dam, Mertens, and Sinkeldam 1994). The most abundant taxa also included *Halamphora minutissima*, *Pinnularia joculata*, and *Rhopalodia operculata*. Ecological preferences of *H. minutissima* are not yet well-known. It is a marine species according to AlgaeBase (Guiry and Guiry 2017) and its type locality is the Po River in Italy (Wetzel et al. 2015). *Pinnularia joculata* was found in high temperature acid sites (Manguin 1942; Quintela et al. 2013). It was reported in the aquatic environments of tropical America (Novelo, Tavera, and Ibarra 2007), but also occurs in Europe, particularly in association

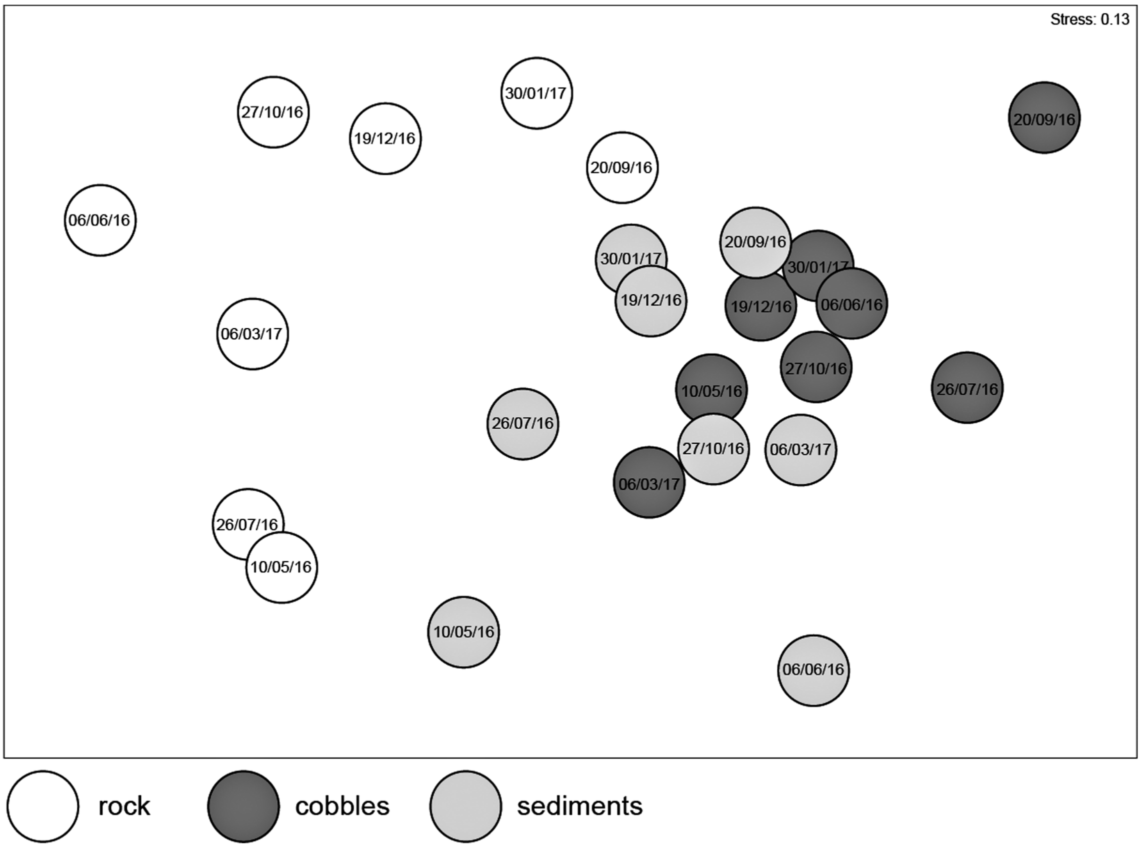


Figure 80. Non-Metric Multidimensional Scaling (nMDS) ordination plot based on species composition of diatom assemblages from the three substrates (rock, cobbles and sediments).

Table 3. Results of analyses of similarity (ANOSIM) evaluating global and pairwise differences of the species assemblages in the three substrates.

Groups	R Statistic	*p* significance level
Global effect	0.554	0.001
Pairwise tests:		
Cobbles vs Sediments	0.397	0.001
Rock vs Cobbles	0.780	0.001
Rock vs Sediments	0.569	0.002

Table 4. Synthesis of results for the SIMPER analysis in the three substrates (average dissimilarity level, number of taxa which contributed to the differences and their cumulative contribution at the cut-off level of 70%).

Groups	Average dissimil. %	N° of taxa	Contrib. cum. %
Cobbles vs Sediments	32.67	17	71.77
Rock vs Cobbles	44.60	15	72.67
Rock vs Sediments	39.59	16	72.54

with botanical-garden greenhouses (Krammer 2000). In the Casteldoria spring, the presence of this species is coherent with the high water temperature and acid pH values (6.3–6.9) measured in some months. Finally, *R. operculata* was reported in mineral-rich environments with high temperatures from different geographic areas such as Canada and Iceland (Villeneuve and Pienitz 1998), Japan (Watanabe et al. 2011), India (Bhakta, Das, and Adhikary 2016), and Spain (Leira, Meijide-Failde, and Torres 2017).

The diatom assemblages of Casteldoria spring showed similarity in species composition with other thermal springs of the Mediterranean area. This comparison revealed 33 taxa (24% of the total), 19 taxa (14% of the total), and 47 taxa (35% of the total) in common with the thermal-sulphur waters of Fiume Caldo in Sicily (Mannino 2007), thermal-sulphur springs of Triponzo in Umbria (Dell'Uomo 1986), and the thermal springs of Grecia (Economou-Amilli 1976), respectively. Overall, 12 taxa (9%) were common with thermo-mineral springs of Katlanovska Banja in the Republic of Macedonia (Stavreva-Veselinovska and Todorovska 2010). However, this comparison is not exhaustive because the study did not report a complete list of the taxa found.

Species composition and differences among substrates

The percentage composition in species of diatom assemblages was fairly stable over time for each substrate. In fact, the five most abundant species selected were present in each substrate in all seasons, albeit with variations in their relative abundance. Differences among assemblages from the three substrates within each season were observed both in relative abundance and the presence/absence of species. Only *Aulacoseira granulata*, *Nitzschia*

Table 5. Values of the Spearman's rank correlation coefficient (*p*) for diatom assemblages from rock. Significant correlations are reported in bold.

	T° C	Q	Cl^-	TP	$N\text{-}NO_3^-$	Rsi	TSS	Br^-	SO_4^{2-}
AAMB	0.072	−0.347	−0.190	−0.381	−0.024	**−0.759***	**−0.790***	−0.156	−0.422
CDUB	−0.127	−0.554	0.072	0.024	−0.060	−0.606	**0.867****	−0.361	−0.703
CMEN	0.248	−0.192	−0.436	−0.246	−0.109	−0.179	**−0.864***	−0.261	**−0.787**
HMNT	0.084	−0.012	−0.333	0.167	**0.833***	0.133	0.252	−0.036	−0.024
NERI	**0.909****	0.608	**−0.755***	−0.287	−0.252	0.400	−0.145	0.355	−0.036
NITZ sp.2	0.277	−0.072	−0.714	**−0.738***	0.143	0.289	−0.036	−0.311	0.060
NMIC	0.663	0.323	**−0.738***	−0.571	−0.262	0.458	−0.036	0.036	0.096
NPAL	0.570	0.711	−0.240	0.228	0.240	−0.006	0.295	**0.741***	0.309
NPRP	0.621	**0.850***	−0.546	−0.273	0.027	0.276	0.302	0.659	0.331
NSRO	0.404	0.338	−0.355	−0.076	−0.089	**0.802***	0.198	−0.006	0.186
SNEO	0.000	−0.515	−0.143	−0.048	0.048	−0.530	**−0.934****	−0.383	**−0.807***

Notes: *= $p<0.05$; **= $p<0.01$; AAMB = *Aulacoseira ambigua*, CDUB = *Cyclostephanos dubius*, CMEN = *Cyclotella meneghiniana*, NERI = *Navicula erifuga*, NMIC = *Nitzschia microcephala*, NPAL = *Nitzschia palea*, NPRP = *Nitzschia perspicua*, NITZ = *Nitzschia* sp., NSRO = *Nitzschia* aff. *subrostrata*, SNEO = *Stephanodiscus neoastraea*.

Table 6. Values of the Spearman's rank correlation coefficient (*p*) for diatom assemblages from cobbles. Significant correlations are reported in bold.

	T° C	Q	pH	Cond	Cl^-	$N\text{-}NO_3^-$	Br^-	Ca^{2+}	Na^+
AAMB	−0.506	**−0.886****	−0.311	−0.524	0.000	0.214	**−0.970****	0.434	0.429
CMEN	0.337	0.371	**0.743***	0.167	0.167	**−0.952****	0.311	0.294	−0.286
CPED	−0.012	0.144	−0.072	0.667	−0.143	−0.238	0.216	**−0.779***	−0.286
DCOF	−0.594	**−0.855***	0.090	−0.659	0.323	−0.060	**−0.747***	0.263	0.719
LGOE	0.188	0.446	0.349	0.263	0.120	**−0.790***	0.524	−0.199	−0.395
NCLA	−0.218	−0.199	0.386	**−0.850***	0.371	−0.108	−0.181	0.559	0.707
NMIC	0.479	**0.765***	−0.229	0.611	−0.275	−0.012	0.590	−0.128	**−0.826***
NPAL	**0.843***	0.695	0.263	0.381	−0.643	−0.214	0.419	0.319	−0.524
PJOC	**0.747***	**0.970****	0.168	0.643	−0.357	−0.238	**0.898****	−0.217	**−0.738***
SNEO	**−0.783***	−0.683	−0.156	−0.524	0.714	0.119	−0.395	−0.089	0.500
SSGE	0.745	0.480	0.041	0.327	**−0.791***	0.191	0.288	0.102	−0.300

Notes: * = $p<0.05$; **= $p<0.01$; AAMB = *Aulacoseira ambigua*, CMEN = *Cyclotella meneghiniana*, CPED = *Cocconeis pediculus*, DCOF = *Diadesmis confervacea*, LGOE = *Luticola goeppertiana*, NCLA = *Nitzschia clausii*, NMIC = *Nitzschia microcephala*, NPAL = *Nitzschia palea*, PJOC = *Pinnularia joculata*, SNEO = *Stephanodiscus neoastraea*, SSGE = *Sellaphora saugerresii*.

Table 7. Values of the Spearman's rank correlation coefficient (*p*) for diatom assemblages from sediments. Significant correlations are reported in bold.

	T° C	Q	pH	Rsi	TSS	Br^-	Ca^{2+}	SO_4^{2-}	Fe^{2+}
CDUB	0.120	−0.371	−0.395	0.253	**−0.731***	−0.587	0.562	**−0.843***	−0.286
CLCT	0.467	0.181	0.120	−0.503	**−0.771***	0.271	0.199	−0.461	−0.719
CMEN	−0.157	−0.563	−0.563	0.120	−0.659	**−0.731***	0.485	−0.723	−0.095
NCLA	−0.648	−0.211	**−0.759***	0.289	0.287	−0.274	−0.170	−0.103	0.685
NERI	−0.313	0.287	−0.443	0.386	**0.862****	0.156	−0.473	0.639	**0.786***
NITZ	0.120	0.683	−0.036	0.181	**0.826***	0.683	−0.562	0.699	0.381
NPAL	0.527	**0.783***	0.024	0.618	0.386	0.476	−0.051	0.267	0.048
NPRP	−0.042	0.012	0.096	**0.776***	0.578	−0.223	−0.051	0.170	0.479
NRCS	0.257	−0.376	0.089	0.347	−0.638	−0.600	**0.816***	−0.578	−0.507
NSRO	**−0.841***	−0.357	−0.466	0.372	0.485	−0.459	−0.034	0.263	**0.799***
PJOC	−0.096	−0.240	**0.743***	0.012	−0.024	−0.216	0.294	0.193	−0.143
PSBR	−0.036	−0.467	0.252	−0.518	**−0.850***	−0.287	0.409	−0.602	−0.571
ROPE	0.229	−0.012	0.455	**−0.819***	−0.192	0.299	−0.358	0.169	−0.333
SNEO	−0.108	−0.551	0.347	−0.482	**−0.766***	−0.371	0.409	−0.518	−0.500

Notes: * = $p<0.05$; **= $p<0.01$; CDUB = *Cyclostephanos dubius*, CLCT = *Caloneis lancettula*, CMEN = *Cyclotella meneghiniana*, HMNT = *Halamphora minutissima*, NCLA = *Nitzschia clausii*, NERI = *Navicula erifuga*, NITZ = *Nitzschia* sp., NPAL = *Nitzschia palea*, NPRP = *Nitzschia perspicua*, NRCS = *Navicula recens*, NSRO = *Nitzschia* aff. *subrostrata*, PJOC = *Pinnularia joculata*, PSBR = *Pseudostaurosira brevistriata*, ROPE = *Rhopalodia operculata*, SNEO = *Stephanodiscus neoastraea*.

sp. and *Stephanodiscus neoastraea* were observed in all substrates in each season. However, the presence/absence of different taxa on the same type of substrate showed a constant pattern over time, suggesting a possible preference of microhabitats. Stable diatom assemblages in thermal springs can reflect a relative temporal stability of abiotic conditions of these aquatic environments. Similar findings were also reported by Quintela et al. (2013). The percentage composition in centric diatoms showed seasonal differences both in assemblages from each substrate and from the three substrates. Overall, cobbles and sediments seem more suitable substrates than rock for centric diatoms. Their highest abundance on these substrates seem related to the greater availability of water along the spring-fed rivulet. In addition, the seasonal oscillations of the water level and of the total suspended solids content, seem to have influenced their distribution, favouring the centric forms in the cobbles in summer (when the discharge is less and the concentration of suspended solids increases) and in the sediments in winter (when the discharge is greater and the concentration of suspended solids decreases).

The diatom assemblages from rock formed a separate cluster from assemblages from cobbles and sediments. Differences between these assemblages, could be due to the different temperature measured at the water emergence point (67.8–71.7°C) and the spring-fed rivulet (54.5–63.8°C). *Pinnularia joculata* and *R. operculata*, which provided the greatest contributions to the differences in both cases, are well adapted to high temperatures, and were always most abundant in assemblages from rock.

Although significant differences in species composition among diatom assemblages from the investigated substrates were found, the traditional approach used in this study, without distinction between living cells and dead frustules and valves, reduced the possibility of an ecologically-sound characterization of the three considered categories.

Relationships between diatom species and environmental variables

The total suspended solids content showed wide oscillations in the spring, attributable to the irregular discharge. In general, the discharge seems to affect diatom life-forms in the biofilm, disadvantaging planktonic species, characterized by a reduced mobility and favouring some free-moving *Navicula* and *Nitzschia* species. The discharge, although always very low, also showed oscillations due to the irregular rainfall regime and the strong water abstraction. It seems to have a similar effect to total suspended solids, being positively correlated with *Nitzschia perspicua*, *N. microcephala* and *N. palea* in the three substrates (respectively rock, cobbles and sediments) and negatively correlated with *Aulacoseira ambigua* in the cobbles. Diatom life-forms are indeed responses to different selective pressures and can explain the necessity of benthic diatoms to adapt to a variety of substrates (Rimet and Bouchez 2012; Round, Crawford, and Mann 1990). Overall, five species were correlated with the water temperature: *Navicula erifuga* in the rock, and *Nitzschia palea* and *Pinnularia joculata* in the cobbles positively, *Stephanodiscus neoastraea* in the cobbles and *Nitzschia* aff. *subrostrata* in the sediments negatively. The influence of temperature on the composition of the diatom communities was highlighted by other studies (e.g. Leira, Meijide-Failde, and Torres 2017; Owen, Renaut, and Jones 2008; Quintela et al. 2013). Finally, significant correlations between diatoms and bromine were observed in all three substrates. The most significant correlations were observed for *A. ambigua* (negative) and *P. joculata* (positive) in the cobbles. Bromine is one of the ions that more characterize the chemistry of the spring, probably resulting from a mixing process with seawater and from the hydrolysis reactions of aluminosilicates (Caboi et al. 1988; Nuti et al. 1977).

Overall, several physical and chemical stressors, such as low discharge, high temperature and high salinity of water, could explain the presence of diatom teratological forms (e.g. Falasco et al. 2009) in the studied spring. When considering the ions more responsible for the high mineralization of water, Na^+ for example is involved in many biochemical processes (Chan, Reyes-Prieto, and Bhattacharya 2011; Sullivan 1976), including the silica uptake, and high concentrations can be toxic (Masmoudi et al. 2013).

Conclusions

The Casteldoria spring was characterized by a high species richness on all investigated substrates, despite several environmental selective pressures. Diatom communities showed similarities in species composition with other Mediterranean thermo-mineral springs, and the most abundant taxa have ecological preferences consistent with the high temperature and mineralization of water. Among these *Pinnularia joculata*, and *Rhopalodia operculata* can be considered representative taxa of the studied spring. This investigation contributes to the knowledge of diatoms in the thermo-mineral springs of Sardinia, providing information on species composition at the small-spatial and seasonal scale. It underlines the importance of sampling the different substrates available in order to document the diatom microflora of spring environments in a more exhaustive way, as well as the need to use specific study approaches for a proper characterization of their assemblages. Further studies should be conducted to broaden the knowledge of these peculiar spring habitats and better understand the relationships between species and environmental factors. The availability and implementation of this information is an important prerequisite for a sound planning of the sustainable use and preservation of these habitats.

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11. Chapter VII

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Epilithic diatom assemblages and environmental quality of the Su Gologone karst spring (central-eastern Sardinia, Italy)

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Abstract – Karst springs are considered among the most vulnerable groundwater-dependent ecosystems. Despite their ecological value and importance as strategic water sources, Mediterranean karst springs are still poorly investigated. The aim of this study was to analyse the epilithic diatom assemblages and to test their usefulness as indicators of environmental quality on the Su Gologone spring (central-eastern Sardinia, Italy), a biotope of great natural value and a precious source of drinking water. A total of 89 diatom taxa were found with 25 new records for Sardinian running waters. Species richness, Shannon-Wiener and Pielou indices showed good biotic integrity. The dominant taxa were alkaliphilous, halophobous-oligohalobous exigent, xeno-oligosaprobic and characteristic of oligotrophic waters. The eutrophication/pollution index − diatom based (EPI-D) and the *Navicula Nitzschia Surirella* indices indicated respectively an excellent/good biological water quality and a low physical disturbance. However, the biological and chemical oxygen demand, and the microbiological variables (*E. coli*, fecal and total coliforms) revealed an organic contamination of the water, although moderate. The judgment provided by the EPI-D should be verified after updating of the index. In fact, 10 taxa found in this study are not currently considered by the EPI-D method.

Keywords: Bacillariophyta, biological quality, diatom indices, groundwater-dependent ecosystems, karst springs, Mediterranean region, physical disturbance, Sardinia

Introduction

Springs are aquatic habitats with unique characteristics and a high ecological value (Odum 1971, Cantonati 2003, Cantonati et al. 2006). They belong to the group of groundwater-dependent ecosystems (GDEs) (Kløve et al. 2011) and provide contacts and connections between groundwater, surface water and terrestrial ecosystems (Webb et al. 1998, Scarsbrook et al. 2007, Cantonati et al. 2012a, b). Their nature of multiple ecotones creates a complex mosaic structure of different microhabitats (Weigand 1998) that makes them important hotspots of biodiversity (Cantonati et al. 2006, Scarsbrook et al. 2007, Ilmonen et al. 2012). Springs are considered insular biotopes (Mac Arthur and Wilson 1967, Whittaker et al. 2001) or water islands (Werum 2001) capable of hosting specific biocenoses because of their disjointed distribution within the landscape (Cantonati et al. 2012b). In addition, they show a greater stability of physico-chemical parameters than other surface aquatic ecosystems (Van der Kamp 1995, Glazier 1998). When pristine or still relatively sheltered from heavy human impacts, they are an important source of high quality water and can host endemic, rare, threatened and relict taxa (Botosaneanu 1995, Cantonati et al. 2006). Springs are among the most interesting aquatic environments for the study of algal microflora, especially diatoms, since they are often the dominant algae and are considered useful indicators of environmental quality, because they can reflect the ecological integrity of spring habitats (Cantonati and Lange-Bertalot 2010, Smol and Stoermer 2010).

The diatom flora of springs from south Europe was investigated in Spain (Aboal et al. 1998, Penalta-Rodríguez and López-Rodríguez 2007, Delgado et. al. 2013), Pyrenees (Sabater and Roca 1990, 1992), Slovenia (Menegalija and Kosi 2008), Bosnia and Herzegovina (Hafner 2008, Kapetanović

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and Hafner 2007), Republic of Macedonia (Stavreva-Veselinovska and Todorovska 2010), Greece (Economou-Amilli and Anagnostidis, 1981).

Studies on the diatom flora of Italian springs have been carried out, especially in recent years, and in particular in the Alpine region (Dell'Uomo 1975, Cantonati 1998a, b, 1999, 2001, 2003, Cantonati and Ortler 1998, Cantonati and Pipp 2000, Battegazzore et al. 2004, Falasco et al. 2012, Cantonati et al. 2006, 2012a, Cantonati and Spitale 2009, Angeli et al. 2010, Falasco and Bona 2011, Spitale and Cantonati 2011, Battegazzore 2012, Battegazzore and Morisi 2012, Spitale et al. 2012a, b) and in the Apennines (Dell'Uomo 1986, Dell'Uomo and Torrisi 2000, 2001, Torrisi and Dell'Uomo 2001, 2009). By contrast, very few studies have investigated springs of the Mediterranean region and, in particular, of the main islands, such as Sicily (Mannino 2007) and Sardinia (Lange-Bertalot et al. 2003). The diatom flora of karst springs, for example, has been explored only occasionally (Dell'Uomo 1990), despite these aquatic ecosystems are particularly important for the Mediterranean area (Civita 2008). Furthermore, this geographic area is one of the major hotspot of plant biodiversity (Myers et al. 2000, Zachos and Habel 2011). Karst springs are considered among the most vulnerable GDEs for both water quantity and quality (Leibundgut 1998). They respond quickly to heavy rainfall and drought periods (Meyer et al. 2003), which are typical of the Mediterranean climate, with wide variations in discharge (White 1988). Their load of suspended solids frequently varies with the discharge (Herman et al. 2007) and can increase significantly during the rainfall period, producing siltation events (Weigand 1998). Karst springs are also increasingly exposed to water abstractions and are particularly sensitive to pollution due to rapid infiltration, thin or absent soil cover, high flow velocity of the water and poor self-purification capacity of the karst aquifer (Sasowsky and Wicks 2000, Daly et al. 2002). These factors can significantly influence the composition and structure of their aquatic communities (Smith et al. 2003, Danehy and Bilby 2009).

Because of the natural scarcity of permanent surface freshwater (Fadda and Pala 1992), in Sardinia karst springs represent a precious and strategic source of drinking water. They are almost the exclusive source of drinking water for many urban centres (De Waele and Murgia 2001). Some studies have emphasized the importance of gaining a greater knowledge and understanding of the biocenoses and ecological dynamics of karst springs in Sardinia, also considering the significant potential vulnerability of these ecosystems (De Waele and Murgia 2001, De Waele 2003). Diatoms, as bioindicators, can provide important information on the environmental integrity of these ecosystems. Moreover, the geographic and ecological isolation of Sardinia, located in the middle of the Mediterranean Sea, is recognized as an important prerequisite for the potential presence of endemic diatom species (Lange-Bertalot et al. 2003).

This study focused on the Su Gologone spring, which is the most important spring in Sardinia, whose algal flora was investigated at the end of the 80s (Dell'Uomo 1990). The main objectives were: a) to describe the current taxonomic composition and structure of the epilithic diatom assemblages of the spring and compare them with data from the previous study; b) to document the presence of interesting taxa by light and scanning electron microscopy; c) to assess for the first time the environmental quality of the spring on the basis of physico-chemical and microbiological parameters and diatom indices.

Materials and methods

Study area

The Su Gologone spring is the most important spring system of Sardinia (Bianco 1993). It is the main resurgence of the Supramonte massif, a vast and complex karst system that extends in central-eastern Sardinia (Fig. 1) (De Waele 2008). The hydrogeological basin that feeds the spring system covers a total surface area of about 160 km^2 and is composed of Middle Jurassic-Upper Cretaceous dolostones and limestones covering a crystalline Palaeozoic basement made out of granites and metamorphic rocks. It is an aquifer of regional importance with a hydrodynamic behaviour that is still in need of proper understanding (De Waele 2008). The Su Gologone spring system is relatively isolated geographically due the surrounding impervious and largely inaccessible mountains. It is inside the Gennargentu and the Gulf of Orosei National Park (Presidential decree 30/03/1998), which, however, has never been operative due to the opposition of the local communities. The Su Gologone is also a Natural Monument (Regional decree 845/1998) and a Respect Zone (Legislative decree 152/1999). The potential sources of disturbance in this territory are mainly local animal farming and hiking activities.

The spring system (Fig. 1) is located in the Guthiddai Valley at the foot of the north-eastern slope of Mount Uddè (806 m) and is composed of two points of water emergence, Sa Vena Manna, a limno-rheocrene spring (104.5 m a.s.l.) and Sa Vena, a rheocrene spring (103.7 m a.s.l.). Sa Vena

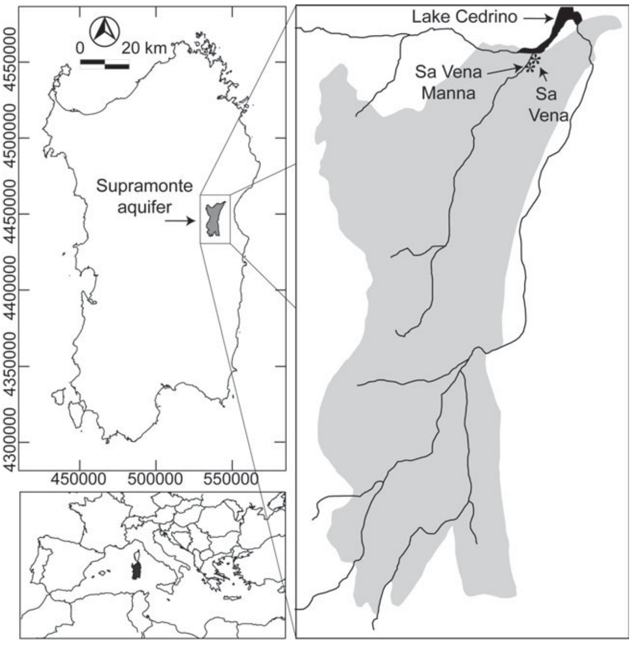

Fig. 1. Geographic location of the Su Gologone karst springs.

Manna, is the largest point of water emergence (Bianco 1993) and is a typical vauclusian spring (De Waele 2008). The water gushes permanently from a fracture in the limestone rock and forms at the opening a narrow and deep pool, trapped between two high and almost vertical rock walls. The water flows slowly in the initial portion and faster in the following stretch. The discharge is high but very irregular, rarely less than 200 L s^{-1} in the drought period (Bianco 1993) and up to 10 000 L s^{-1} during rainfalls (De Waele 2008). Sa Vena is the smallest of the two water emergences of the Su Gologone karst system and has a much more modest water discharge. The water emerges from a small rock from which it is abstracted by a small aqueduct (Bianco 1993) and immediately forms a small brook. Water abstraction from Sa Vena supplies drinking water to the municipalities of Oliena and Dorgali (about 16 000 inhabitants). Waters from both Sa Vena Manna and Sa Vena flow, after a short distance, into the Cedrino River and further downstream into Lake Cedrino, a eutrophic artificial lake, which supplies water to the downstream municipalities (about 20 000 inhabitants) (Bianco 1993, Padedda and Sechi 2008). When floods of the Cedrino River occur (on average twice a year), the spring system is submerged by the waters of the lake for a time ranging from a couple of hours to several days, making it impossible to supply drinking water (Bianco 1993, De Waele 2008).

Sampling

The sampling was carried out at Sa Vena between December 2010 and June 2011. Water samples for chemical and microbiological analyses were collected each month using 1 L polyethylene and glass bottles. The water samples were preserved in cold and dark conditions until the laboratory analyses were performed. Epilithic diatoms were collected in December 2010 and June 2011 by scraping the upper surface of hard natural substrates (five cobbles randomly selected in flowing water for a total surface of at least 100 cm^2) with a hard bristled toothbrush according to the methods of Kelly et al. (1998) and Ispra (2007). All diatom samples were preserved in 100 mL polyethylene bottles and fixed in situ with a formaldehyde solution (4% v/v).

Measurements and analyses

Temperature, pH, conductivity and dissolved oxygen were measured in situ with a multi-parameter probe (YSI MPS 556). Alkalinity, chlorides (Cl^-), hardness, biological oxygen demand (BOD), chemical oxygen demand (COD), soluble reactive phosphorus ($P\text{-}PO_4^{3-}$), total phosphorus (TP), ammonia nitrogen ($N\text{-}NH_4^+$), nitrites ($N\text{-}NO_2^-$), nitrates ($N\text{-}NO_3^-$), total nitrogen (TN), reactive silica (RSi), total suspended solids (TSS) and some ions (Ca^{2+}, Mg^{2+}, Fe^{2+} and Mn^{2+}) were determined in the laboratory according to standard methods reported by CNR-IRSA (1994) and APHA (1998). *Escherichia coli*, fecal and total coliforms were analysed using a membrane filtration method according to the CNR-IRSA (1994).

Diatom subsamples (50 mL) were treated in the laboratory, after natural decantation for 48 h. The organic matter was eliminated by boiling the samples in hydrogen peroxide (30%). Diluted hydrochloric acid (37%) was added to remove carbonates (ISPRA 2007). After being washed with distilled water, the cleaned frustules were mounted on permanent microscope slides using Styrax® resin (refractive index = 1.59).

Diatoms were examined using a Zeiss Axiovert 10 light microscope (LM) at a 1000× magnification. In the first stage, the identification of all species was done according to Krammer and Lange-Bertalot (1986, 1988, 1991a, b, 2000), Lange-Bertalot et al. (2003), Reichardt (2004), Werum and Lange-Bertalot (2004), Levkov (2009), Lange-Bertalot et al. (2011), Żelazna-Wieczorek (2011). Light microscope images were taken with an Axiocam Zeiss digital camera mounted on the microscope and connected to a computer. Afterwards, for each sample, at least 400 valves and/or frustules were counted. For the purpose of corroborating the LM study, several species were also examined using a Zeiss EVO LS10 environmental scanning electron microscope (SEM). Subsamples of the diatom suspension were air-dried on aluminium sheets and fixed on aluminium stubs that were sputter-coated with gold (Sputter Coater Edwards S-150A). The SEM identification of some diatom taxa was made after consultation of the current taxonomic literature available (e.g., Idei and Kobayasi 1986, Reichardt 2009, Van de Vijver et al. 2011, Jovanovska et al. 2013).

Data processing

All species observed in the samples were used to draw up a complete floristic list which was compared with that obtained in a previous study performed on the algal microflora of the Su Gologone spring at the end of the 80s (Dell'Uomo 1990). The ecological preferences of all diatom taxa were investigated primarily by referring to Dell'-Uomo (2004), Torrisi and Dell'Uomo (2009) and Van Dam et al. (1994). In addition, the first indications on the vulnerability degree of the observed taxa were taken from the German Red List of threatened diatoms proposed by Lange-Bertalot and Steindorf (1996). This Red List, although compiled for the local diatom flora and not updated since 1996, is the only international reference currently available for the classification of diatoms based on their vulnerability.

The taxa present in the counts were used for the analysis of structure of the diatom assemblages and the evaluation of the environmental quality and physical disturbance of the spring. The abundance values of diatom taxa in each sample were transformed into relative abundance values which express the percentage contribution of each species compared to the total contribution of all species present in the count of each sample. The relative percentage abundance of each taxon was calculated by dividing the number of valves and/or frustules by the total number of valves and/or frustules of all the taxa counted in each sample and multiplying this quotient by 100.

The biotic integrity of the spring was estimated by calculating species richness, Shannon-Wiener diversity index, 2 based logarithm (H'), and evenness (J') (Shannon 1948, Shannon and Weaver 1949, Pielou 1975).

Synthetic ecological spectra of pH, salinity, organic matter and nutrients were obtained considering both the presence and the relative abundances of the taxa. The taxa with a wider ecological range were placed between the respective autecological levels, dividing equally the values of their relative percentage abundance.

Biological water quality was evaluated using the eutrophication/pollution index − diatom based (EPI-D) (Dell'Uomo 2004). The EPI-D index was chosen because it is the only index developed in Italy, after a long period of research conducted mainly on diatom communities of the central Apennines, but also of the Southern Alps and Apennines. Moreover, this index has already been applied with good results to different Italian springs, including karst springs such as the Clitunno springs (Torrisi and Dell'Uomo 2001). This index, based on the Zelinka and Marvan formula (Zelinka and Marvan 1961), considers the sensitivity (affinity/tolerance) of diatoms to nutrients, organic matter and degree of water mineralization, providing an estimation of the general quality of the water body. The values of the EPI-D were expressed in the original scale from 0 to 4, with values close to 0 indicating excellent quality and values close to 4 indicating very bad quality. The results around the threshold values were considered as transition classes (transition interval ± 0.05).

The degree of physical disturbance (siltation) was inferred by applying the *Navicula Nitzschia Surirella* indices (NNS and NNS') (Battegazzore et al. 2003, 2004, 2007). The NNS and NNS', based on the work by Hill et al. (2001) and Bahls (1993), provide an estimate of the physical disturbance in an aquatic ecosystem due to natural and anthropogenic factors assuming that siltation events determine an increase in the proportion of motile taxa within the community, both in terms of number of taxa and of number of individuals. The NNS (qualitative index) was calculated as a percentage ratio between the number of motile taxa belonging to the genera *Navicula*, *Nitzschia* and *Surirella* and the total number of taxa recorded in each sample. The NNS' (quantitative index) was calculated as a percentage ratio between the number of individuals belonging to three genera *Navicula*, *Nitzschia* and *Surirella* and the total number of individuals recorded in each sample. The values of both indices range from 0 to 100. Values close to 0 represent a low

Tab. 1. Monthly and average values of the physico-chemical and microbiological variables measured and analyzed in the karst spring Su Gologone (Sa Vena). D. L. – detection level; N/A – not available; BOD – biological oxygen demand; COD – chemical oxygen demand; TP – total phosphorus; TN – total nitrogen; TSS – total suspended solids; RSi – reactive silica; UFC – units forming colony.

Variables	D. L.	07/12/10	12/01/11	10/02/2011	07/03/11	18/04/11	18/05/11	13/06/11	Average
Temperature (°C)	-5 °C	12.4	12.0	12.0	12.0	12.0	13.0	13.0	12.3
pH (units)	0	7.6	7.9	8.0	7.8	8.3	8.1	7.5	7.9
Conductivity (µS cm^{-1})	0	356	254	353	336	338	322	344	329
Alkalinity (meq L^{-1})	N/A	2.7	2.7	2.9	2.7	2.6	2.8	2.6	2.7
Dissolved oxygen (mg L^{-1})	0	9.2	10.6	11.6	7.7	11.7	12.2	10.7	10.5
Oxygen saturation (%)	0	86	98	107	72	109	116	73	94
BOD (mg L^{-1})	N/A	3.6	1.6	3.3	0.9	2.9	4.6	2.9	2.8
COD (mg L^{-1})	5	–	< D. L.	< D. L.	18.1	< D. L.	9.2	23.7	17.0
Cl$^-$ (mg L^{-1})	5	21.3	14.2	14.2	12.4	16.0	13.5	17.7	15.6
Hardness (mg L^{-1})	5	120	105	143	133	145	143	155	135
P-PO$_4^{3-}$ (µg L^{-1})	4	4.0	4.0	4.0	< D.L.	4.0	4.0	< D.L.	4.0
TP (µg L^{-1})	4	14.0	38.0	11.0	12.0	13.0	13.0	12.0	16.0
N-NH$_4^+$ (µg L^{-1})	5	14.0	10.0	15.0	10.0	13.0	13.0	33.0	15.0
N-NO$_2^-$ (µg L^{-1})	1	< D. L.	< D. L.	< D. L.	1.0	< D. L.	< D. L.	1.0	1.0
N-NO$_3^-$ (µg L^{-1})	50	554.0	560.0	271.0	388.0	544.0	458.0	551.0	475.0
TN (µg L^{-1})	300	719.0	679.0	762.0	643.0	679.0	769.0	943.0	742.0
RSi (mg L^{-1})	0.05	1.8	2.1	1.8	1.5	2.0	2.0	2.0	1.9
Ca^{2+} (mg L^{-1})	N/A	40.1	40.1	88.0	52.0	44.0	46.0	49.0	51.0
Fe^{2+} (mg L^{-1})	0.001	0.008	0.005	0.011	0.013	0.008	0.007	0.013	0.009
Mg^{2+} (mg L^{-1})	N/A	4.9	1.2	19.0	1.0	8.5	6.6	7.9	6.9
Mn^{2+} (mg L^{-1})	0.01	< D. L.	< D. L.	0.011	0.019	< D. L.	0.010	0.023	0.016
TSS (mg L^{-1})	N/A	3.0	–	1.0	3.0	1.0	13.0	0.4	3.6
Escherichia coli (UFC 100 mL^{-1})	1	38	69	5	2	80	25	158	54
Fecal coliforms (UFC 100 mL^{-1})	1	51	115	20	1	103	170	342	115
Total coliforms (UFC 100 mL^{-1})	1	95	202	25	8	184	210	606	190

level of physical disturbance, whereas values close to 100 indicate a high level of physical disturbance.

All indices applied in this study, except the NNS and NNS', were calculated using the software OMNIDIA 7 V. 8.1 (Lecointe et al. 1993).

Statistical analyses

Principal component analysis (PCA) was performed using R 3.1.3 (Venables et al. 2015) on environmental variables to detect temporal differences among samplings and to better characterize the diatom samplings made in December and June. For the ordination analysis data were normalized using a (x-mean)/standard deviation. In the PCA analysis, we included temperature (Temp), conductivity (Cond), hardness (Hardn), biological oxygen demand (BOD), chlorides (Cl^-), nitrates ($N\text{-}NO_3^-$), ammonia nitrogen ($N\text{-}NH_4^+$), soluble reactive phosphorus ($P\text{-}PO_4^{3-}$).

Differences in the specific composition of the two seasonal diatom assemblages were analysed with a t-test performed on the abundances of the taxa present in each diatom sample, using R 3.1.0 (R CORE TEAM 2012).

Results

Environmental variables

The monthly and average values of the physico-chemical and microbiological variables are reported in Tab. 1. During our study, the water temperature varied from a minimum of 12 °C (January–April) to a maximum of 13 °C (May–June). Waters had a slightly basic pH (7.5–8.3), with an intermediate level of hardness (105–155 mg L^{-1} $CaCO_3$). Ca^{2+} was the most abundant cation and ranged between 40.1 mg L^{-1} (December–January) and 88.0 mg L^{-1} (February). Its highest concentration coincided with the maximum of Mg^{2+} (19.0 mg L^{-1}), which was always much lower in the other months (1.0–8.5 mg L^{-1}). Fe^{2+} and Mn^{2+}were present as trace elements in very small amounts. The conductivity ranged from 254 to 356 µS cm^{-1}. The percent water oxygenation was generally > 75%. Slight supersaturation was observed in February, April and May (107–116). $P\text{-}PO_4^{3-}$ showed values of 4.0 µg L^{-1} in almost all months, while TP values ranged from 11.0 to 38.0 µg L^{-1}. $N\text{-}NO_3^-$ was the most abundant inorganic nitrogen compound (271.0–560.0 µg L^{-1}). TN concentrations were in the range of 643.0–943.0 µg L^{-1}. BOD and COD values showed peaks in May (4.6 mg L^{-1}) and June (23.7 mg L^{-1}). The bacterial load varied widely with higher densities observed in the spring, from April to June. Suspended solids had very low concentrations with the exception of an isolated highest value in May (13 mg L^{-1}).

The PCA on environmental variables showed relationships among variables and between variables and samples (Fig. 2). The first axis explained 38.3% of the variance and the second 23.7%. $N\text{-}NH_4^+$ (0.48), water temperature (0.45) and hardness (0.44) were positively related to the first axis, characterizing the diatom sampling in June. The second axis was associated with $N\text{-}NO_3^-$ (0.54), Cl^- (0.48) and $P\text{-}PO_4^{3-}$ (0.44) at opposite positions, characterizing the diatom sampling in December.

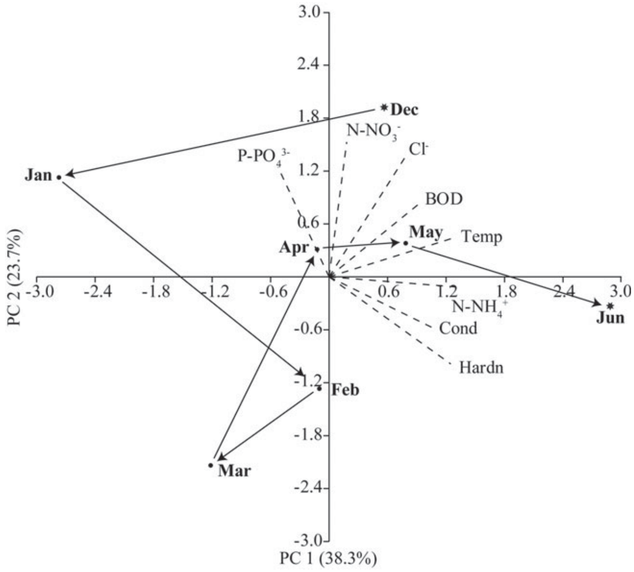

Fig. 2. Principal component analysis (PCA) biplot with the environmental variables. Temp – water temperature; Cond – conductivity; Hardn – hardness; BOD – biological oxygen demand; Cl^- – chlorides; $N\text{-}NO_3^-$ – nitrates; $N\text{-}NH_4^+$ – ammonia nitrogen; $P\text{-}PO_4^{3-}$ – soluble reactive phosphorus. Dec – December; Feb – February; Mar – March; Apr – April; May – May; Jun – June.

Diatom assemblages

The complete list of taxa and their ecological preferences are reported (Tab. 2). The list is composed of a total of 89 diatom taxa, belonging to 36 genera. The genera with the highest number of species were *Navicula* (10), *Nitzschia* (9) and *Achnanthidium* (7), followed by *Amphora*, *Cocconeis*, *Diploneis* and *Gomphonema* (6). According to Van Dam et al. (1994), the majority of the species observed mainly occur in water bodies. Only 9 taxa (10% of the total) are not strictly linked to aquatic environments and belong to the categories "nearly exclusively occurring outside water bodies" and "mainly occurring on wet and moist or temporarily dry places". Of the total 89 taxa identified in our study, 52 (58% of the total) are included in different categories of the German diatom Red List (Lange-Bertalot and Steindorf 1996). However, only 5 taxa are classified as "in regression" and 2 taxa "extremely rare".

The diatom assemblages included 25 new records for the Su Gologone spring, and more for Sardinian running waters in general. LM and SEM images of some abundant, frequent, rare and occasional taxa, including some new records, were reported respectively in Figs. 5 A–P and Figs. 6 A–O.

Overall, there were 39 taxa present in the counts (44% of the total), belonging to 20 genera with 1 genus of Centrales (*Ellerbeckia*) and 19 genera of Pennales (38 taxa). They included 6 abundant taxa (relative abundance more than 5%), 10 frequent (relative abundance between 1.5 and 5%) and 23 rare (relative abundance less than 1.5%). *Achnanthidium subatomus*, *Amphora pediculus* and *Achnanthidium minutissimum* were the most abundant taxa in the

Tab. 2. List of the diatom taxa observed in the karst spring Su Gologone (Sa Vena) and their ecological preferences. All the acronyms used for pH, salinity, saprobity and trophic state are explained in the legend of the figure 3. MA – maximum relative abundance in at least one sample: r – rare: < 1.5%, f – frequent: 1.5 – 5%, a – abundant: > 5%; RL – Germain Red List of diatom taxa (Lange-Bertalot and Steindorf 1996): * – currently not considered endangered, ** – surely not endangered, 2 – highly endangered, 3 – endangered, D – insufficient data, G – considered at risk, V – in regression, R – extremely rare; Moisture: 1 – never, or only very rarely, occurring outside water bodies, 2 – mainly occurring in water bodies, sometimes in wet places, 3 – mainly occurring in water bodies, also rather regularly on wet and moist places, 4 – mainly occurring in wet and moist or temporarily dry places, 5 – nearly exclusively occurring outside water bodies. In bold: new diatom records for the Su Gologone karst spring. Taxa highlighted in gray color are not currently included in the eutrophication/pollution index − diatom based method.

Taxa	1985–1986	2010–2011	MA	RL	pH	Salinity	Saprobity	Trophic state	Moisture
Achnanthes coarctata (Brébisson) Grunow	X	X		* *	n	hb-oe	x-o	hypo-oligo	5
Achnanthidium acsiae Wojtal, E. Morales, Van de Vijver & Ector		X		–	–	–	–	–	–
Achnanthium affine (Grunow) Czarnecki	X			*					
Achnanthidium bioretii (H. Germain) O. Monnier, Lange-Bertalot & Ector		X		V	n	hb-oe	x-o	hypo-oligo	4
Achnanthidium exiguum (Grunow) Czarnecki		X		* *	ak	oe-ot	o-β	oligo-meso	3
Achnanthidium lineare W. Smith	X	X		–	n	–	–	–	–
Achnanthidium minutissimum sensu lato (Kützing) Czarnecki	X	X	a	–	n-ak	oe	o	oligo	3
***Achnanthidium* sp.**		X		–	–	–	–	–	–
Achnanthidium subatomus (Hustedt) Lange-Bertalot		X	a	*	–	–	–	–	–
Amphipleura pellucida (Kützing) Kützing		X	r	*	ak	oe	o-β	oligo-meso	2
Amphora indistincta Levkov		X		*	–	–	–	–	–
Amphora meridionalis Levkov		X		–	–	–	–	–	–
Amphora ovalis (Kützing) Kützing	X	X	r	* *	ak	ot	o-β	oligo-meso	1
Amphora pediculus (Kützing) Grunow ex A. Schmidt	X	X	a	* *	ak	ot	x-β	oligo-meso	3
Amphora vetula Levkov		X		–	–	–	–	–	–
Asterionella formosa Hassall		X		* *	ak	oe-ot	o-β	oligo-meso	1
Caloneis bacillum (Grunow) Cleve	X			* *					
Caloneis fontinalis (Grunow) Cleve-Euler		X	f	–	–	–	–	–	–
Caloneis lancettula (Schulz) Lange-Bertalot & Witkowski		X		–	–	–	–	–	–
Campylodiscus hibernicus Ehrenberg	X			*					
Cocconeis euglypta Ehrenberg	X	X	r	–	ak	ot-h	o-α	oligo-eu	2
Cocconeis neothumensis Krammer		X		V	akb	–	o	–	–
Cocconeis placentula sensu lato Ehrenberg	X	X	f	–	ak	ot	x-β	oligo-meso	2
Cocconeis placentula var. *lineata* (Ehrenberg) van Heurck	X	X		* *	ak	ot	x-β	oligo-meso	2
Cocconeis placentula var. *placentula sensu* Jahn et al. 2009		X		* *	–	–	–	–	–
Cocconeis pseudolineata (Geitler) Lange-Bertalot		X	f	D	ak	ot	x-β	oligo-meso	–
Cyclotella ocellata Pantocsek	X			*					
Cymbella affinis Kützing		X		*	ak	hb-os	o	oligo	2
Cymbella helvetica Kützing	X			V					
Cymbella lanceolata (Ehrenberg) Van Heurck	X			V					
Cymbopleura cuspidata (Kützing) Krammer	X			V					
Denticula tenuis var. *crassula* (Nägeli) Hustedt	X			V					
Diatoma hyemalis (Roth) Heiberg	X			*					
Diatoma mesodon (Ehrenberg) Kützing	X	X	r	*	n	hb-oe	o	oligo	2
Diploneis elliptica (Kützing) Cleve		X		*	a-k	oe	x-o	hypo-oligo	3
Diploneis minuta J.B. Petersen		X		R	–	–	–	–	5
Diploneis* cf. *oculata		X	f	–	–	–	–	–	–
Diploneis oculata (Brébisson) Cleve	X			*					
Diploneis separanda Lange-Bertalot		X	r	–	–	–	–	–	–
***Diploneis* sp. 1**		X	r	–	–	–	–	–	–
***Diploneis* sp. 2**		X		–	–	–	–	–	–

Tab. 2. – continued

Taxa	1985–1986	2010–2011	MA	RL	pH	Salinity	Saprobity	Trophic state	Moisture
Ellerbeckia arenaria (Moore ex Ralfs) R.M. Crawford	X	X	r	* *	a-k	hb	o	hypo	4
Encyonema elginense (Krammer) D.G. Mann	X			2					
Encyonema silesiacum (Bleisch) D.G. Mann		X		*	n	ot	β	meso	1
Encyonema ventricosum (C. Agardh) Grunow	X	X		*	n	oe	o	oligo	–
Encyonema vulgare Krammer		X		V	–	–	–	–	–
Eolimna minima (Grunow) Lange-Bertalot		X	r	* *	ak	h	α	eu	3
Epithemia argus (Ehrenberg) Kützing	X			*					
***Epithemia* sp.**		X		–	–	–	–	–	–
Eunotia mucophila (Lange-Bertalot, Nörpel & Alles) Lange-Bertalot	X			G					
Eunotia pectinalis (Kützing) Rabenhorst		X	r	V	ac	hb-oe	x-o	hypo-oligo	3
Eunotia sp.		X			–	–	–	–	–
Eunotia valida Hustedt	X			–					
Fallacia mitis (Hustedt) D.G. Mann		X	r	–	ak	oe	x	oligo	–
Fallacia subhamulata (Grunow) D.G. Mann		X	f	*	n	oe	o-β	meso	3
Fragilaria capucina Desmazières		X	r	–	n-ak	oe	o	oligo	–
Fragilaria mesolepta Rabenhorst	X			* *					
Fragilaria recapitellata Lange-Bertalot & Metzeltin		X		–	n	oe	o-β	meso	–
Frustulia vulgaris (Thwaites) De Toni		X		* *	ak	oe	o-β	meso	3
*Geissleria decussis (*Østrup) Lange-Bertalot & Metzeltin		X		* *	ak	oe	o	oligo	3
Gomphonema acuminatum Ehrenberg	X			* *					
Gomphonema angustius E. Reichardt		X		–	–	–	–	–	–
Gomphonema clavatum Ehrenberg		X		*	n-i	hb	x-o	oligo	3
Gomphonema elegantissimum E. Reichardt & Lange-Bertalot		X	r	–	–	–	–	–	–
Gomphonema micropus Kützing		X		*	ak	α-meso	β	eu	–
Gomphonema productum (Grunow) Lange-Bertalot & E. Reichardt	X			D					
Gomphonema pumilum (Grunow) E. Reichardt & Lange-Bertalot	X			*					
Gomphonema pumilum var. *rigidum* E. Reichardt & Lange-Bertalot		X		–	–	–	–	–	–
Gomphonema truncatum Ehrenberg	X	X		*	ak	ot	o-β	oligo-meso	–
Gomphosphenia grovei var. *lingulata* (Hustedt) Lange-Bertalot		X		–	–	–	–	–	–
Gyrosigma sciotense (Sullivant & Wormley) Cleve	X								
Halamphora montana (Krasske) Levkov		X		*	ak	oe	o-β	meso	4
Humidophila contenta R.L. Lowe et al.		X		* *	ak	oe-ot	o-β	oligo-meso	4
Karayevia clevei (Grunow) Bukhtiyarova		X	r	*	ak	oe	o-β	oligo-meso	1
Karayevia ploenensis var. *gessneri* (Hustedt) Bukhtiyarova		X	r	–	–	–	–	–	–
Luticola goeppertiana (Bleisch) D.G. Mann		X	r	* *	ak	h-β	α-p	eu-hyper	3
Melosira varians C. Agardh	X	X		* *	ak	ot	x-α	oligo-eu	2
Meridion circulare (Greville) C. Agardh	X	X	f	* *	ak	hb-oe	x-o	oligo	1
Navicula antonii Lange-Bertalot		X	r	* *	–	–	–	–	–
Navicula chiarae Lange-Bertalot & Genkal		X		–	–	–	–	–	–
Navicula cincta (Ehrenberg) Ralfs		X		* *	ak	h	α	eu	4
Navicula cryptocephala Kützing	X			* *					
Navicula cryptotenella Lange-Bertalot	X	X	f	–	ak	oe	o-β	oligo-meso	2
Navicula cryptotenelloides Lange-Bertalot		X	r	* *	ak	–	–	–	–
Navicula gregaria Donkin		X	r	* *	ak	h	α	meso	3
Navicula menisculus Schumann	X			V					
Navicula reichardtiana Lange-Bertalot		X		–	ak	oe	o-β	oligo-meso	–
Navicula tripunctata (O.F. Müller) Bory	X	X	a	* *	ak	hb-oe	o	oligo	3

Tab. 2. – continued

Taxa	1985–1986	2010–2011	MA	RL	pH	Salinity	Saprobity	Trophic state	Moisture
Navicula radiosa Kützing	X			* *					
Navicula veneta Kützing		X	r	* *	ak	h-β	α-p	eu-hyper	3
Navicula vilaplanii (Lange-Bertalot & Sabater) Lange-Bertalot & Sabater		X		R	–	–	–	–	–
Nitzschia commutata Grunow		X	r	*	–	–	–	–	–
Nitzschia dissipata (Kützing) Grunow	X	X	f	–	ak	ot	o-α	meso	3
Nitzschia fonticola (Grunow) Grunow	X	X	f	–	ak	oe	o-β	oligo-meso	1
Nitzschia frustulum (Kützing) Grunow		X		–	ak	ot-h	β-α	meso-eu	3
Nitzschia inconspicua Grunow		X	r	* *	ak	ot-h	β-α	meso-eu	3
Nitzschia linearis (C. Agardh) W. Smith	X	X	r	–	ak	ot-h	β-α	meso-eu	3
Nitzschia recta Hantzsch ex Rabenhorst		X		–	ak	ot	β	meso	1
Nitzschia sigmoidea (Nitzsch) W. Smith	X			* *					
Nitzschia sociabilis Hustedt		X	f	* *	n	ot-h	β-α	meso-eu	1
Nitzschia solgensis Cleve-Euler		X		V	ak	oe-ot	o-β	oligo-meso	4
Placoneis clementis (Grunow) E.J. Cox		X		*	ak	oe-ot	o-β	oligo-meso	3
Planothidium delicatulum (Kützing) Round & Bukhtiyarova	X			*					
Planothidium ellipticum (Cleve) M.B. Edlund	X			–					
Planothidium frequentissimum (Lange-Bertalot) Lange-Bertalot		X	a	–	ak	hb-oe	α-poli	ind	–
Planothidium lanceolatum (Brébisson ex Kützing) Lange-Bertalot	X	X	a	–	ak	hb-oe	o	oligo	3
Platessa hustedtii (Krasske) Lange-Bertalot		X		*	ak	hb-oe	o	oligo	4
Pleurosigma elongatum W. Smith	X			*					
Reimeria sinuata (W. Gregory) Kociolek & Stoermer		X		* *	n	oe	o	oligo	3
Reimeria uniseriata S.E. Sala, J.M. Guerrero & Ferrario		X		–	–	–	–	–	–
Rhoicosphenia abbreviata (C. Agardh) Lange-Bertalot	X	X	r	* *	ak	oe	o-β	oligo-meso	2
Sellaphora sp.		X		–	–	–	–	–	–
Simonsenia delognei (Grunow) Lange-Bertalot		X		* *	–	ot	α	eu	3
Staurosira construens Ehrenberg		X			ak	oe	o	oligo	1
Ulnaria acus (Kützing) Aboal	X			*					
Ulnaria ulna (Nitzsch) Compère	X	X		*	ak	ot	β	meso	2

winter sample (respectively, 28%, 23% and 14%). Prevailing in the spring sample were *A. pediculus*, *A. subatomus* and *Planothidium frequentissimum* (26%, 15% and 9% respectively %).

The results of the biotic integrity indices are reported in Tab. 3. Species richness varied from 25 in winter to 31 in late spring and the diatom samples showed 20 taxa that were common to both seasons. The values of the Shannon-Wiener diversity index and the Pielou index (evenness) were respectively 3.27 and 0.70 in winter and 3.69 and 0.75 in late spring.

Tab. 3. Seasonal values of the biotic integrity indices applied to diatom assemblages of the karst spring Su Gologone (Sa Vena).

	Species richness	Diversity (H')	Evenness (J')
December 2010	25	3.27	0.70
June 2011	31	3.69	0.75

The t-test, performed on the abundances of the taxa present in each sample, indicated significant differences between the diatom assemblages in winter and late spring ($p < 0.01$).

Synthetic ecological spectra of pH, salinity, organic matter and nutrients are reported in Figs. 3A–D. The pH spectrum (Fig. 3A) showed a dominance of alkaliphilous species (69%), while circumneutral diatoms were present in insignificant amounts (4%). In the salinity spectrum (Fig. 3B), the halophobous and oligohalobous exigent (38%) prevailed over the oligohalobous tolerant diatoms (32%) that tolerate moderate concentrations of dissolved salts and, in particular, of chlorides. The halophilous forms that prefer water with a higher salt content were present in a negligible quantity (2%). The saprobic spectrum (Fig. 3C) revealed a greater presence of xenosaprobic and oligosaprobic taxa (43%) in respect to the β-mesosaprobic species (18%). The presence of the α-mesosaprobic (7%) and polysaprobic species (4%), which require a higher quantity of organic compounds, was negligible. With regard to preferences for the

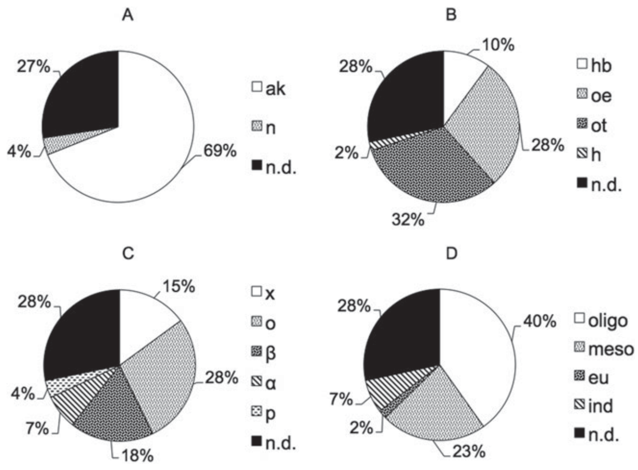

Fig. 3. Synthetic ecological spectra of the diatom taxa observed in the karst spring Su Gologone (Sa Vena): A) pH: ak – alkaliphilous, n – circumneutral; B) salinity: hb – halophobous, oe – oligohalobous exigent, ot – oligohalobous tolerant, h – halophilous; C) saprobity: x – xenosaprobic, o – oligosaprobic, β – β-mesosaprobic, α – α-mesosaprobic; p – polysaprobic; D) trophic state; taxa characteristic of environment: oligo – oligotrophic, meso – mesotrophic, eu – eutrophic; ind – indifferent taxa; for all spectra: n.d. – no data (ecological preferences poorly known).

trophic characteristics of the water bodies (Fig. 3D), the species characteristic of oligotrophic waters (40%) were most abundant in respect to the species characteristic of mesotrophic waters (23%). The eutraphentic taxa, characteristic of more nutrient-rich water bodies, were insignificant (2%) as were indifferent taxa with a wide ecological range (7%).

Diatom indices

The EPI-D values in winter (0.99) and in late spring (1.01) revealed an excellent/good water quality (class I–II) in both seasons (Fig. 4).

The NNS and NNS' indices revealed a low physical disturbance both in winter and in late spring. In particular, the NNS index showed a slightly greater number of mobile

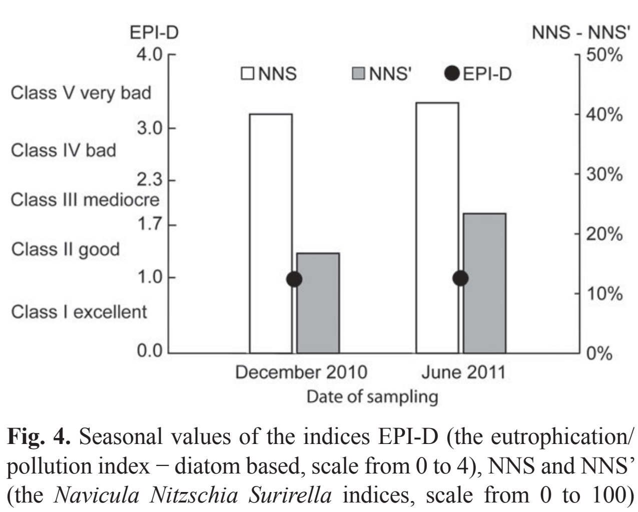

Fig. 4. Seasonal values of the indices EPI-D (the eutrophication/pollution index − diatom based, scale from 0 to 4), NNS and NNS' (the *Navicula Nitzschia Surirella* indices, scale from 0 to 100) used for the evaluation of the biological water quality and physical disturbance of the karst spring Su Gologone (Sa Vena).

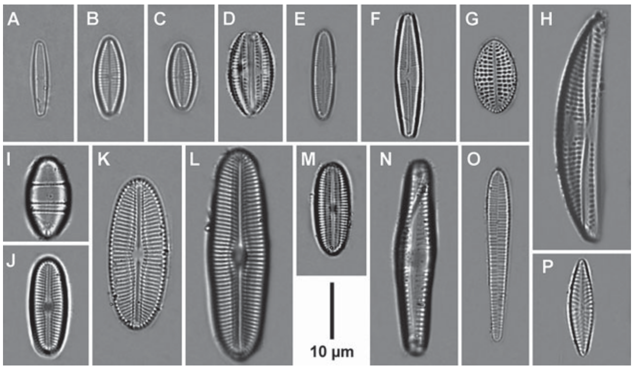

Fig. 5. Light microscopy. A) *Achnanthidium lineare* W. Smith; B-C) *Achnanthidium subatomus* (Hustedt) Lange-Bertalot; D) *Amphora indistincta* Levkov; E) *Caloneis fontinalis* (Grunow) Cleve-Euler; F) *Caloneis lancettula* (Schulz) Lange-Bertalot & Witkowski; G) *Cocconeis neothumensis* Krammer; H) *Amphora meridionalis* Levkov; I) *Diatoma mesodon* (Ehrenberg) Kützing; J) *Diploneis separanda* Lange-Bertalot; K) *Diploneis* sp. 1; L) *Diploneis* sp. 2; M) *Diploneis minuta* J.B. Petersen; N) *Gomphonema angustius* E. Reichardt, initial valve; O) *Meridion circulare* (Greville) C. Agardh; P) *Navicula vilaplanii* (Lange-Bertalot & Sabater) Lange-Bertalot & Sabater in Rumrich et al.. Scale bar = 10 µm.

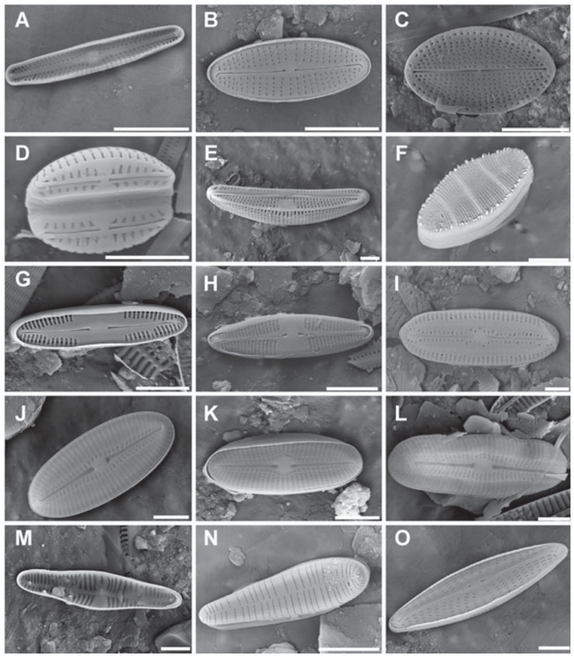

Fig. 6. Scanning electron microscopy. A) *Achnanthidium lineare* W. Smith, internal view of raphe valve; B) *Achnanthidium subatomus* (Hustedt) Lange-Bertalot, external view of raphe valve; C) *Cocconeis neothumensis* Krammer, external view of raphe valve; D) *Amphora indistincta* Levkov, external view; E) *Amphora vetula* Levkov, external view; F) *Diatoma mesodon* (Ehrenberg) Kützing, external view; G) *Caloneis fontinalis* (Grunow) Cleve-Euler, external view; H) *Caloneis lancettula* (Schulz) Lange-Bertalot & Witkowski, external view; I) *Diploneis minuta* J.B. Petersen, external view; J) *Diploneis* sp. 1, external view; K) *Diploneis separanda* Lange-Bertalot, external view; L) *Diploneis* sp. 2., external view; M) *Gomphonema angustius* E. Reichardt, internal view of initial valve; N) *Meridion circulare* (Greville) C. Agardh, external view; O) *Navicula vilaplanii* (Lange-Bertalot & Sabater) Lange-Bertalot & Sabater in Rumrich et al., external view. Scale bars = 5 µm.

taxa in spring (41.9%) than in winter (40.0%). Also the NNS' index showed a slightly higher number of individuals belonging to the mobile genera in late spring (23.4%) than in winter (16.7%).

Discussion

Despite its small dimensions, the Su Gologone spring at Sa Vena hosts a diatom flora with high total species richness, reflecting a high diversity of microhabitats and a moderate level of anthropic disturbance. The moderate current, due to the mild slope of the terrain, may have contributed to the high number of species found. In fact, according to many authors, the fast current generally present in the rheocrene springs may favour the rheophilous taxa, determining a loss in species number and diversity (Sabater and Roca 1992, Cantonati 1998a, 2001, Cantonati et al. 2012a). Moreover, rheocrene springs on carbonate substrate seem to be generally characterized by epilithic diatom communities with a lower diversity of species than rheocrene springs on siliceous substrate (e.g., Cantonati 1998a, Gesierich and Kofler 2010). The total species richness (89 taxa) was higher than that observed in the previous investigation carried out at the end of the 80s (49 taxa) (Dell'Uomo 1990), suggesting a possible role of the protective measures present since 1998 in the studied area. In fact, in other geographic areas, like for example the Alpine region, the species richness found in protected areas is higher than in non-protected areas (e.g., Falasco and Bona 2011).

The comparison between our list of species and that reported by Dell'Uomo (1990) indicated considerable differences in the Su Gologone spring after 25 years, with the presence of 21 taxa in common in both studies, 29 taxa detected only in the previous study and 68 taxa observed only in this study. The latter included 25 new records for the spring and more in general for running waters of Sardinia. The differences found seem mostly due to advances of taxonomic knowledge because several taxa like *Navicula antonii*, *Navicula vilaplanii*, *Diploneis separanda*, *Gomphonema angustius*, *Amphora indistincta*, *Amphora meridionalis*, *Amphora vetula*, were described some years after the study carried out in 1985–1986. Except for the temperature, previous environmental data of the spring are not available. However, differences found could be also related with environmental changes. In fact, species like *Campylodiscus hibernicus*, *Cymbella helvetica*, *Encyonema elginense*, *Cyclotella ocellata*, *Navicula radiosa*, (xenosaprobic and characteristic of hypotrophic or ultraoligotrophic environments) were not detected during our investigation, and nor were *Planothidium ellipticum* and *Diatoma hyemalis*, the only two taxa belonging to the nordic-alpine flora (Dell'Uomo 1990). In the central Apennines, *D. hyemalis* is reported as a species "at risk of extinction". It seems to be increasingly rare and exclusively located at high altitudes, in cold well-oxygenated waters, and with a fast current (Torrisi and Dell'Uomo 2009). A longer study could ascertain the real state of *P. ellipticum* and *D. hyemalis* in the Su Gologone spring, also taking into account a possible effect of global warming on these cold water stenotherm species. Instead, the new records found are probably explicable by the lack of specific studies on communities of benthic diatoms in the running waters of Sardinia.

In accordance with the results of the previous study, the diatom species of the Su Gologone spring have mostly a cosmopolitan and boreal-mountain distribution. The diatom assemblages showed some common features with some Alpine springs, like the presence of abundant and frequent taxa, such as *Achnanthidium minutissimum*, *Meridion circulare* and *Planothidium lanceolatum* (Cantonati 1998a, Battegazzore et al. 2004, Gesierich and Kofler 2010, Falasco and Bona 2011) and a high number of subdominant and rare taxa (e.g., Cantonati 1998a, Gesierich and Kofler 2010). However, a comparison with the floristic lists from other Italian springs showed a higher number of species in common with the diatom communities found in different springs and headwaters of rivers with the calcareous substrate found in the central Apennine region (e.g., Torrisi and Dell'Uomo 2009). Several taxa were also in common with diatom communities of springs in the Castellón province (Aboal et al. 1998), Pyrenean springs (Sabater and Roca 1992) and spring-fed streams on Majorca Island (Delgado et al. 2013).

Overall, the spring hosted a small group of taxa that are not closely linked to the aquatic environment according to Van Dam et al. (1994). For example, *Achnanthes coarctata* and *Diploneis minuta* were reported as species occurring nearly exclusively outside water bodies. Although this group of species accounted for 10% of the total species observed, they are part of the biodiversity of the spring and underline its nature of ecotone.

The vulnerable species according to the German Red List were *Achnanthidium bioretii*, *Cocconeis neothumensis*, *Encyonema vulgare* and *Nitzschia solgensis*, considered species "in regression", *D. minuta* and *N. vilaplanii* included as "extremely rare". All these species were occasional members of the diatom communities of the spring. This information has only a purely indicative and preliminary value for Sardinia. In fact, data on distribution and abundance of diatom taxa are recent and still scarce and prevent an extended comparison with the inland waters of the island. However, these first indications could integrate those from other regions and provide a contribution to a first possible diatom Red List for the Italian territory.

The indices of biotic integrity revealed high species richness and diversity and a balanced distribution of the species in the current diatom assemblages. The species richness and diversity were higher in late spring, probably due to a greater degree of light irradiation. In permanent springs with a constant temperature, the light regime is an important factor for the seasonal changes in the algal communities (e.g., Ward and Dufford 1979). Spring is also reported to be the season with the highest diatom diversity (e.g., Cantonati 1998a).

The t-test analysis indicated significant seasonal changes in the diatom assemblages, unlike what was reported for example for the Alpine springs on a carbonate substrate (Cantonati 1998a).

The observed diatom assemblages were dominated by *Achnanthidium subatomus* in winter and *Amphora pediculus* in late spring. As to *A. subatomus*, no information is available about its preference for organic matter, while with respect to its trophic preferences Krammer and Lange-Bertalot (1991b) indicated that it privileges oligo-mesotrophic waters. *Amphora pediculus* is a xeno-β-mesosaprobic species and prefers oligo-mesotrophic environments. Other dominant species were *A. minutissimum*, *Navicula tripunctata*, *Planothidium frequentissimum*, *Caloneis fontinalis*, *Nitzschia dissipata* and *P. lanceolatum*. Among these species, *N. dissipata* is an oligo-α-mesosaprobic species typical of mesotrophic environments, whereas *P. frequentissimum* is an α-meso-polisaprobic species and colonizes environments with different trophic states (Van Dam et al. 1994). All other species are oligosaprobic and typical of oligotrophic waters, except for *C. fontinalis*, about which no autecological information is available. In contrast, *Fragilaria mesolepta*, a species xeno-oligosaprobic and typical of hypo-oligotrophic waters, dominated the diatom assemblages in a previous study (Dell'Uomo 1990). The ecological preferences of the dominant species in the current assemblages suggest a possible slight deterioration of the water quality, especially of the organic type, over the years.

In general, the synthetic ecological spectra highlighted the dominance of species that prefer alkaline waters and low to moderate concentrations of dissolved salts, organic matter and nutrients. These results were very consistent with those of the physico-chemical analyses and EPI-D index. In fact, the water was characterized by a slightly basic pH and a medium hardness and degree of water mineralization. With respect to the nutrient concentrations, in particular of phosphorus, the Su Gologone spring may be considered an ecosystem with a low trophic level, which is comparable with that of the Alpine springs (Cantonati 1998a). However, their values and in particular nitrate values, do not reflect the condition of pristine environments. Moreover, BOD and especially COD, and microbiological variables indicated a not negligible degree of organic contamination in certain months. It is potentially attributable to pasture activities carried out in the surrounding area, the pollutant load of the Cedrino River and the periodic submersion of the spring by the Cedrino Lake. The highest densities of both *Escherichia coli* and fecal and total coliforms were observed in the spring season, probably due to the reduction of the discharge. This kind of contamination, although moderate, is important for the water quality, especially in view of its use as drinking water.

The EPI-D index revealed an excellent/good biological water quality (class I–II) in both seasons. However, a slight deterioration of the biological quality was observed in late spring, probably as a result of some peaks of BOD and COD in the months before the diatom sampling (June). The judgment provided by the EPI-D index seems fairly reliable and probably only slightly overestimates the quality of water. In our study *Eolimna minima* and *Navicula gregaria*, species strictly α-mesosaprobic and *Nitzschia inconspicua* and *Nitzschia sociabilis*, species β-α-mesosaprobic, showed very low relative abundances (0.1–1.6%). Among α-polysaprobic species *P. frequentissimum* was the taxon with higher relative abundance (5.1% in winter and 9.1% in late spring). The EPI-D index integrates the sensitivity of each species to salinity, organic matter and nutrients and it mediates the sensitivity of each species among different environmental variables. It provides a global judgment on the quality of the water body reflecting the interactions of several variables (Dell'Uomo 2004), rather than just organic matter. The PCA analysis performed on the environmental variables also indicated that the BOD was less important than other variables in December and June and may have affected to a lesser extent the structure of the diatom assemblages. However, 10 taxa found in this study (26% of the total), including *P. frequentissimum,* currently are not considered by the EPI-D method and their values of "i" and "r" are not present in the Omnidia software. For this reason, our result should be checked after the update of the EPI-D index in order to obtain a more accurate assessment. The NNS and NNS' indices indicated a low degree of physical disturbance, in accordance with the low concentrations of suspended solids observed throughout the period of the study. Both indices indicated a slight worsening in late spring, following an anomalous peak of the concentration of suspended solids in May. These results were also consistent with the low physical disturbance reported for the Su Gologone spring on the basis of the Disturbance Index for Karst environments (KDI) (De Waele 2009).

This study has contributed to a greater understanding of the diatom flora of the Su Gologone spring, which is the most important of Sardinia. It has led to an initial ecological characterization of the diatom assemblages, albeit limited to winter and late spring (wet period). Moreover it is the first work on the use of epilithic diatoms as indicators of biological quality and physical disturbance in the karst springs of Sardinia. Despite the presence of some potential impacts in the territory, the Su Gologone spring showed a high environmental quality, probably only slightly overestimated by the EPI-D index, and a good level of biotic integrity, reflecting the scarce human presence and the very high degree of naturalness of the hydrogeological basin. The high species richness and the presence of diatom taxa included in the German Red List also underline the importance of protecting and preserving this important biotope. However, the periodic submersion of the spring due to floods of the Cedrino River should be assessed more accurately in order to evaluate the role of floods as stressors for the ecosystem and their aquatic communities. In addition, the organic contamination detected in this study, albeit moderate, remains a problem that should no longer be neglected. The biocenoses and ecological dynamics of the Sardinian karst springs are still largely unknown. Yet these peculiar environments deserve greater consideration because of their high natural value and vulnerability and their strategic importance as sources of drinking water. Further investigations will contribute to the gathering of useful information for their long-term monitoring and management.

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12. General conclusions

This thesis gives new contribution to knowledge of karst and thermo-mineral springs of Sardinia, so far mostly limited to hydrogeological, physical and chemical aspects. The research activities carried out, allowed to document the biodiversity of diatoms from different springs, previously unexplored, and to acquire a wide range of information on the ecological characteristics of these ecosystems. The case studies presented highlighted some main aspects:

a) high biodiversity

Diatom assemblages showed a high species richness despite the presence of several natural and anthropogenic pressures (small size, strong climate seasonality, high temperatures and mineral content, nutrient inputs and water abstraction). This aspect suggests the role of these environments as hotspots of biodiversity at the regional level. The study focused on species is an interesting field of investigation that allows to highlight local specificities and contributes to a more accurate ecological characterization of the spring systems. Taxonomic insights carried out with the support of expert botanists, allowed to describe *Sellaphora gologonica sp. nov.* from the karst spring Sa Vena (Su Gologone system). A second study allowed to document the first record of *Chamaepinnularia thermophila* in Sardinia (thermo-mineral spring San Giovanni Su Anzu) and, more in general, in the Mediterranean area. An accurate morphological comparison with *Navicula tongatensis* from the Hustedt's original material revealed identical features that led to suggest their synonymy. An interesting aspect of this species is its occurrence in very few sites in the world, especially springs in islands. In fact, springs are considered in themselves "water islands" and the mechanisms of dispersion for diatoms are passive and limited;

b) similarities and differences with other geographic regions

The floristic lists of the springs studied showed similarities with those reported in literature for different geographic areas, especially for the Mediterranean area. Karst springs, in particular, showed a high number of species common to springs and headwaters with calcareous substrate of the central Apennine region.

By contrast, the direct comparison based on the analysis of similarity among assemblages from thermo-mineral springs of Sardinia and Auvergne (France), showed considerable differences in species composition despite the possible common geological history of these two geographic regions. These results support the role of different environmental factors in the distribution of species;

c) heterogeneity of sites

The occurrence of several species in only one site confirmed the wide heterogeneity of spring systems also from the biological point of view. Assemblages from karst springs showed a greater similarity in species composition than thermo-mineral springs. The significant differences found in geothermal sites clearly reflected the different water chemistry, already documented in historical studies. Similarly to other studies, these differences did not allow to establish characteristic combinations of species. However, some abundant taxa, well adapted to specific conditions such as temperature and mineral content, or common to multiple sites, can be considered representative of these environments;

d) vulnerability to natural and human pressures

Most of the studied springs showed evident alterations due to inadequate water abstraction systems. In some cases, the original morphology was strongly modified by the construction of closed housing and water channeling and natural flows were greatly reduced. Disturbance due to trampling and handling of cobbles was also observed in different sites, mainly in the thermal springs that are used for free bath. Furthermore, abandoned waste, were occasionally found in different sites. Overall, the nutrient contents did not reflect the condition of pristine environments, indicating the influence of activities mainly related to agriculture and animal breeding in the catchment areas. A study carried out on the karst spring Su Gologone also revealed changes in environmental conditions and diatom assemblages after the extratropical cyclone Cleopatra in November 2013, highlighting the further threat of extreme events due to climate change;

e) relevance of diatoms in ecological surveys

Diatoms showed significant correlations with different environmental variables, recognized as important drivers for growth and distribution of species in many studies (e.g., temperature, pH and conductivity). They were able to reflect specific abiotic conditions of the different sites as well as their vulnerability related to important factors like nutrient enrichment and variations in hydrological conditions due to the irregular rainfall regime, climate change and water abstraction. The study of diatoms is a promising topic of investigation which can be useful in the development of specific tools for assessing the environmental quality of spring ecosystems;

f) methodological aspects

Research activities carried out in this thesis revealed at least three significant points from a methodological point of view: (i) the sampling of diatoms from different substrates allow to document diatom biodiversity in a more exhaustive way and to investigate the different response capacities of the assemblages to environmental factors; (ii) water discharge is an important factor influencing assemblages in springs and should be measured with reliable tools in larger systems in future studies; (iii) specific study approaches capable of distinguishing living cells and dead frustules and valves should be used for an ecologically-sound characterization of diatom assemblages from different substrates in thermal springs.

The identification of diatoms is a difficult step that requires considerable effort and time. Several taxa found, sometimes abundant, were not identified to the species level during this Ph.D. In addition, the numerous data collected allow further processing for future insights. This thesis offers food for thought on the importance of enhancing and preserving these environments so important from an ecological point of view and of so large interest for human uses. The information collected may represent a starting point for the development of strategies aimed at a proper management.

The knowledge of ecological aspects is an important prerequisite for a sound planning of the sustainable use and preservation of springs. In fact biological communities play a key role in the functioning of these systems and can provide important information on their environmental integrity. Moreover, only what is known can be managed properly and the approaches used can be considered effective only if they are aimed at maintaining all components of these ecosystems.

In Sardinia, included in the list of major “climate change hot spots” springs have strategic importance and their valorization and management should be a priority objective.

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