The threat of groundwater pollution for petrifying springs; defining nutrient threshold values for an endangered bryophyte community. V8_22-03-2021 (total. 5876 words excl Ref.)

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Abstract:

Eutrophication by human activities is increasingly affecting ecosystem functioning and community composition. So far, studies focus on the effects of atmospheric nitrogen deposition, surface water eutrophication or soil nutrient accumulation. Groundwater pollution of spring habitats, however, has received much less attention, although numerous papers report groundwater nutrient enrichment worldwide. This study presents the results of a survey on groundwater pollution (with emphasis on nitrate and ortho-phosphate concentrations) and bryophyte composition in 51 ambient petrifying springs in 5 NW-European countries, which were compared to published data from 173 other sites from 11 European countries.

The dataset covers a broad range of unpolluted to heavily polluted springs with nitrate concentrations between 0.7 and 3227 µmol l^{-1}. Most NW-European petrifying springs were found to have groundwater pollution (nitrate and phosphate) with the most polluted petrifying springs occurring in The Netherlands. The cover of individual characteristic bryophyte species significantly correlates with groundwater nutrient concentrations indicating that nutrient pollution of spring waters affects the bryophyte composition. *Palustriella commutata, Eucladium verticillatum* and *Brachythecium rivulare* prefer unpolluted petrifying springs where as *Cratoneuron filicinum* and *Pellia endiviifolia* show a much broader tolerance to groundwater pollution. For ecohydrologically healthy petrifying springs threshold values of 288 µmol (18 mg l) NO_3^- l^{-1} and 0.42 µmol (0.04 mg l^{-1}) ortho-PO_4^{2-} l^{-1} were defined.

Data analysis of the spring water composition indicates that the main source for nutrient enrichment are nitrate and base cation losses from intensively used agricultural fields. This results in various anthropogenic induced chemical processes in subsoil and aquifers that can result in the pollution of groundwater and subsequently damage petrifying spring habitats.

Further regulations for nitrate and phosphate application are required in order to conserve and restore groundwater fed ecosystems in Europe.

Keywords: ambient springs, *Cratoneurion*, EU-Habitat Directive, base cation leaching, nutrient threshold values.

INTRODUCTION

Anthropogenic nutrient enrichment

Nutrient availability (both nitrogen (N) and phosphorus (P)) is one of the most influential abiotic factors determining ecosystem functioning and community composition and is strongly promoted by anthropogenic influences including surface water eutrophication and atmospheric N deposition (Conley et al., 2009; Bobbink et al., 2010). In oligotrophic and mesotrophic habitats N and P are the primary limiting nutrients for plant growth leading to detrimental effects when nutrient availability increases (e.g. (~~Bobbink et al., 1998~~; Wassen et al., 2005; Bobbink et al., 2010; Ceulemans et al., 2014).

Characteristic species in these habitats are adapted to nutrient-poor conditions and can only compete with other faster growing species and survive under low nutrient availability (Aerts & Chapin III, 1999). Effects of enhanced N and P loads have been studied in a wide variety of ecosystems, i.e. forests (Dise & Wright, 1995; Nordin et al., 2005), heathlands (Aerts et al., 1990; Gordon et al., 2001), grasslands (Duprè et al., 2010; Stevens et al., 2010), raised bogs (Limpens, et al., 2004; Tomassen et al., 2004; Fritz et al., 2012) and dunes (Remke et al., 2009; Kooijman et al., 2017). These studies reveal that the effects of nutrient enrichment on ecosystem functioning and biodiversity are often complex and may differ on both temporal and spatial scales (e.g. Bobbink et al., 2010; Ceulemans et al., 2014). However, the consequences of nutrient enrichment on the functioning and species composition of groundwater fed spring habitats has received only minor attention. Most of the studies on anthropogenic nutrient availability focus on the effects of atmospheric N deposition (REF?), surface water eutrophication (REF?) or soil nutrient enrichment (REF?). Nutrient concentrations at groundwater fed spring ecosystems can be expected to be more important due to high nutrient loads over time compared to other systems.

Since the 1970s, increased groundwater nitrate (NO_3^-) concentrations have become a significant and increasing environmental issue worldwide (Thorburn et al., 2003; Burow et al., 2010; Howden et al., 2011; Rivett et al., 2008). Increasing global agricultural activity and intensity has resulted in an increased NO_3^- leaching to the groundwater (Van Diggelen et al., 1995; Di & Cameron, 2002; Gundersen et al., 2006).

Petrifying springs

Petrifying springs with travertine (tufa) formation are found globally across a variety of environments including; alkaline fens, calcareous grassland, spring forests, peatlands and open countryside, and over a range of altitudes (Hájek et al., 2002; Pentecost, 2005; Cantonati et al., 2016; Dobrowolski et al., 2016). Active travertine accumulating petrifying springs dominated by bryophytes are generally small but range from several square metres with a thickness of only a few cm up to 20 hectares with a height of 492 meters (Sivá Brada, Slovakia; (Grootjans & Gojdičová, 2011). The vegetation of active petrifying springs is dominated by bryophytes belonging to *Cratoneurion commutati* alliance. Characteristic species of this community are: *Cratoneuron filicinum, Brachythecium rivulare, Fissidens taxifolius, Eucladium verticillatum* and *Palustriella* *commutata* (previously named *Cratoneuron commutatum*)*,* the latter very often the most abundant species (Heery, 2007).

Active petrifying springs with a well-developed bryophyte community are rare and endangered in Europe. The European Union has assigned the habitat type "7220 Petrifying springs with travertine formation *(Cratoneurion)*" to the list of protected European habitat types and declared it a priority habitat type under the European Natura 2000 Habitat Directive (EU-HD, 1992). As for other plant communities it is generally considered that nutrient enrichment of petrifying springs might negatively influence the functioning and community composition of these sensitive ecosystems. It is also known that increasing soluble phosphate concentrations may even limit the petrifying process itself (Lin & Singer 2006; Zeppenfeld, 2019). As little knowledge still exists on the degree and exact nature of nutrient pollution at petrifying spring habitats it remains unclear which measures must be taken to conserve or restore these systems.

Groundwater influence

Petrifying springs are groundwater dependent ecosystems, as their biota and physical structure are strongly influenced by groundwater supply and quality. Up until 2010 the information on water chemistry of these springs in relation to bryophyte communities was limited to some individual locations (Hartkopf-Fröder et al., 1989; Pentecost, 1996; Pentecost & Zhaohui, 2002; Hájek et al., 2002; Columbu et al., 2013). Most publications dealing with petrifying systems concern primarily geochemistry and paleo-ecological development (Coûteaux, 1969; Ford & Pedley, 1996; Hoffman, 2005; Pentecost, 2005; Thomas, 2007; Cantonati et al., 2016). The diatom flora of petrifying springs in relation to groundwater composition is studied by (Arp et al., 2010; Denys & Oosterlynck, 2015). Since 2010 some local and regional studies have been published which address the interrelationship between bryophyte and spring water composition in more detail (Oosterlynck & De Bie, 2000; Hájková et al., 2012; Farr et al., 2014; Lyons, 2015; Grootjans et al., 2015; Couvreur et al., 2016; Farr & Graham, 2017).

Present study

Here we present a field study and a review of data on NW European petrifying springs in order to unravel the relationship between chemical composition of spring water and bryophyte composition. Data has been colalted from a broad range of severely polluted to almost unpolluted springs.

We hypothesize (1) that enhanced groundwater nutrient concentrations (nitrate and phosphate) influence the bryophyte community in a negative way and lower the abundances of characteristic bryophyte species of petrifying springs. Based on correlations found we aimed to establish groundwater nutrient threshold values for European petrifying springs.

Furthermore, we hypothesize (2) that groundwater nitrate enrichment influences biogeochemical processes in the aquifer which result in an altered water composition in petrifying springs.

The results will be discussed in relation to future management and the conservation of petrifying springs in Europe.

MATERIALS AND METHODS

Sampling locations and method

In the present study 51 NW European petrifying springs ("*7220* Petrifying springs with travertine formation *(Cratoneurion)*" (Interpretation Manual of European Union Habitats, version EUR 28, 2013) were sampled. These predominantly forested sites are in; The Netherlands (11), Belgium (12), Germany (20), Luxembourg (3) and France (5), (Figure 1), Sampling locations are provided in the supplementary materials).

Both well and poorly developed ambient petrifying springs were included in the study. In well-developed sites the most characteristic species of active petrifying springs are *Palustriella commutata, Brachythecium rivulare, Cratoneuron filicinum, Pellia endiviifolia* and *Eucladium verticillatum* while in poorly developed springs only a few of these species are present. In all sampled sites bryophyte composition and general site conditions are described. Water samples were taken between February and April 2016. At each sampling site a 200 ml surface water sample was collected in a PE bottle close to the spring head or start of the petrified zone. All samples were cooled during transportation and stored under dark and cool conditions (4°C) until analyses. An additional 50 ml water sample was taken to determine alkalinity and pH within 16 hours after sampling under cooled conditions. Alkalinity was determined using a titrimetric

MColortest™ Alkalinity Test (Merck Millipore). The pH was measured using a handheld WTW pH96 meter with a SenTix21 electrode.

Bryophyte composition and species cover was determined in relevees made over a length of 10 - 50 m along a homogeneous part of the alluvial beds of brooks, runnels and seepages including, if present, small petrified tufa dams, using the Braun-Blanquet approach (Braun-Blanquet, 1964). Bryophyte nomenclature follows (Hill et al., 2006; Van Dort et al., 2010).

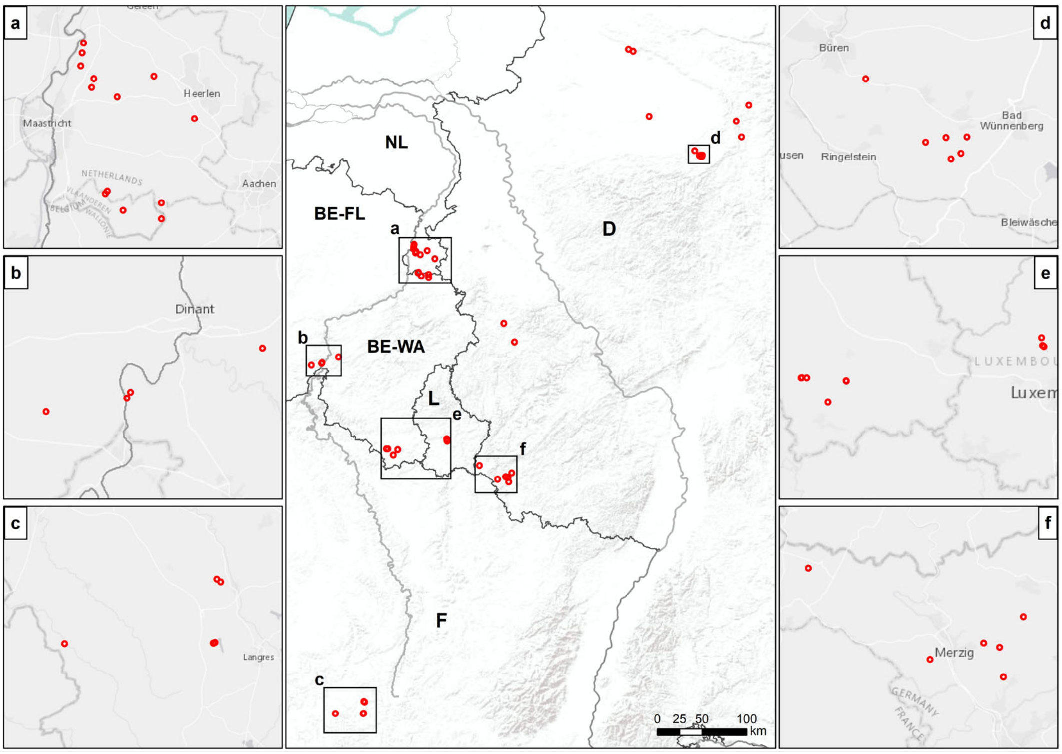

Figure 1: Overview of the 51 locations sampled in The Netherlands (NL), Belgium (BE), Germany (D), France (FR) and Luxembourg (L).

Inorganic chemistry

Prior to elemental analyses, 10 ml of each sample was stored at 4°C until analysis with 0.1 ml (65%) HNO_3^- added to prevent metal precipitation. For analyses of $P_{(tot)}$, Ca, Mg, Fe, $S_{(tot)}$ and Al inductively coupled plasma spectrophotometry (ICP-Optical Emission Spectrometer, Thermo Scientific iCAP 6000 Series ICP) was used. To determine NO_3^- (Kamphake et al., 1967) NH_4^+ (Grasshoff & Johannsen, 1972), ortho-phosphate (PO_4^{2-}) (Henriksen, 1965) and Cl^- concentrations, 20 ml of each sample was stored at -20°C and analysed calorimetrically with an Auto Analyzer 3 system

(Bran+Luebbe). Na^+ and K^+ were determined with a Technicon Flame Photometer IV Control (Technicon Corporation).

Additional data from literature

Existing studies of petrifying spring habitats that contained water chemical analysis were collated and used as reference data. Data of the chemical composition of spring water samples was only included if it met the following criteria:

- The samples were collected in ambient spring systems with well-developed bryophytes vegetation
- The sites were below 500 m a.m.s.l.
- The sites were not influenced by coastal sea spray
- Duplicates in sampling were avoided. If various samples from the same spring system were available, preferably the sample of the most upstream situated sampling point was selected.
- Method of chemical analyses was comparable. Several reported PO_4^{2-} concentrations have been excluded from our analysis for this reason.

In total, inorganic water chemistry data was collated for an additional 173 petrifying springs located in The Netherlands (20) Belgium (36), Germany (5), the United Kingdom (34), Ireland (32) France (4), Poland (24), Latvia (7), Slovakia (10) and Croatia (1). (see also Supplementary Table S1). In most occasions data on NO_3^- were available but not always on PO_4^{2-}. Concentrations are presented both in mols and in miligrams per litre (nitrate and phosphate).

Data analysis

Statistical analysis was undertaken using the statistical program SPSS (IBM statistics, version 21) and R (R Core Team, 2016) base package and vegan package (Oksanen et al., 2013). A standard boxplot was used to depict the variation in water chemistry. Correlations between bryophytes and environmental parameters were tested using a linear regression model.

Redundancy Analysis (RDA) was used as ordination method to visualise the variance in occurring bryophyte composition between the different sites. The RDA was carried out on the five most frequent and characteristic bryophyte species in our survey (*Cratoneuron filicinum, Brachythecium rivulare, Palustriella commutata, Eucladium* *verticillatum* and *Pellia endiviifolia*) and a selection of chemical parameters (Ca^{2+}, Cl^-, EC, HCO_3^-, K^+, Na^+, NH_4^+, NO_3^-, pH, PO_4^{2-} and $S_{(tot)}$).

Threshold values for groundwater nutrient levels for petrifying springs

Based on the data from this study threshold concentrations for NO_3^- and PO_4^{3-} were calculated. Three bryophyte species *Palustriella commutata, Eucladium verticillatum* and *Brachythecium rivulare* were selected based on their relative sensitivity to pollution as shown by their negative correlations with increased nutrients and base cation levels (Figure 4). Subsequently all locations sampled within the present study were classified into three categories based on the presence and coverage of these three critical mosses according to the following criteria;

- poor (impoverished): absence of the three selected critical moss species
- moderate: presence of one to three of the selected moss species and a total coverage less than 50%
- good: presence of one to three of the selected moss species and a total coverage more than 50%

To define threshold values, we follow the approach from the UK Technical Advisory Group on the Water Frame work Directive (UKTAG, 2012): the threshold value must (1) lie below the mean and preferably below the 25th percentile for sites in poor condition, (2) lie above the mean and preferably above the 75th percentile for sites in good condition and (3) reflect available knowledge from literature from within the site research.

RESULTS

Chemical composition

Analysis of water from petrifying springs and water chemistry data collated from theliterature all showed spring water originating from calcareous rich deposits but with great diversity in chemical composition; i.e. the pH ranging from 7-8.5, electrical conductivity from 38 to over 100 mS m^{-1} and Ca^{2+}+Mg^{2+} concentrations ranging from 1500 - 6250 μmol l^{-1}). Other elements like Cl^- and K^+ showed similar variations. The sum of Ca^{2+} and Mg^{2+} showed a significant correlation with NO_3^- concentrations (R^2 0.46, P <0.01) and PO_4^{2-} concentrations (R^2 0.33, P <0.01) (see also Supplementary Fig. S2).

Spring water NO_3^- and PO_4^{2-} concentrations varied between 0.0 to 3227 µmol l^{-1} NO_3^- and 0.002 – 4.4 µmol l^{-1} PO_4^{2-}). NH_4^+ concentrations were usually relatively low (<68.5 µmol l^{-1}, data not shown). Nutrient concentrations in petrifying spring water revealed large differences between countries with the highest NO_3^- levels were found in The Netherlands (Figure 2a; average 1363 NO_3^- µmol l^{-1}) followed by Northern Belgium (Flanders: average 511 µmol NO_3^- l^{-1}) and Luxemburg (average 441 µmol NO_3^- l^{-1}). Average NO_3^- concentrations in Southern Belgium (Wallonia), France, Germany and the United Kingdom were between 210 and 260 µmol NO_3^- l^{-1}. Data from Ireland, Latvia, Poland and Slovakia showed the lowest NO_3^- concentrations (averages between 2 and 100 µmol NO_3^- l^{-1}).

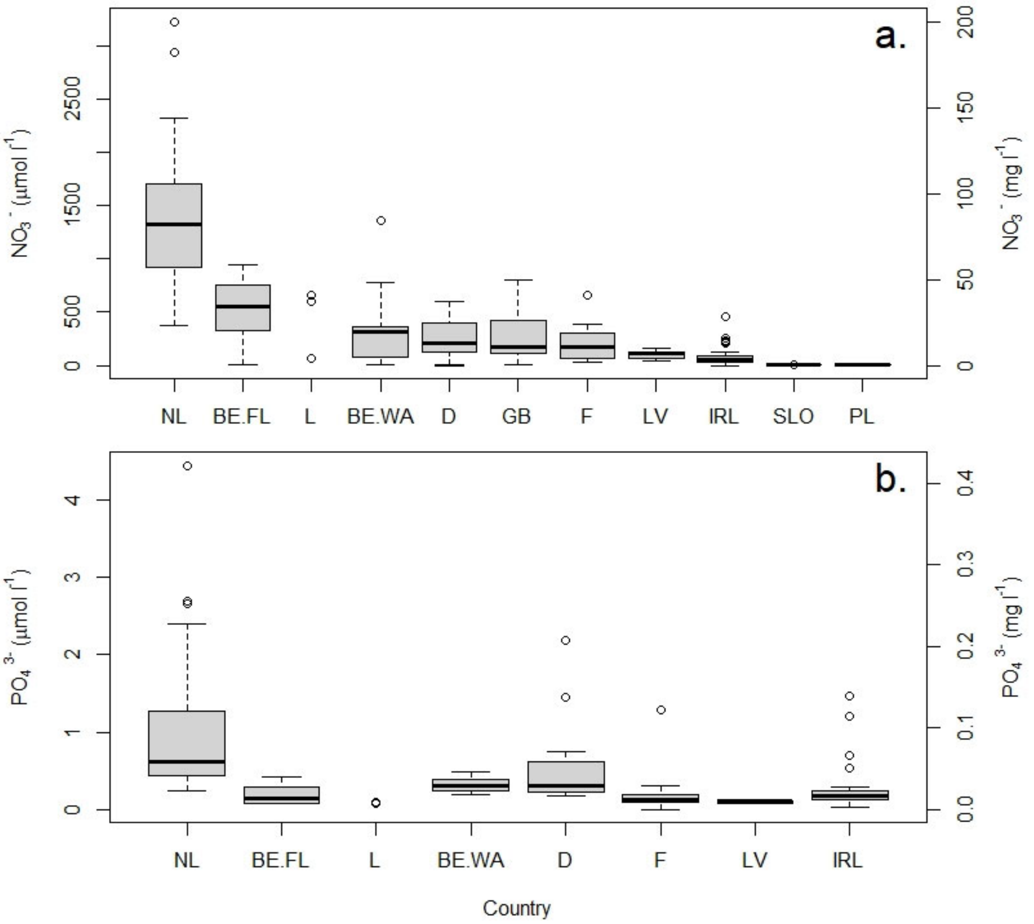

*Figure 2: The NO_3^- and ortho-PO_4^{2-} concentration in petrifying springs presented in boxplots per country (**a**: NO_3^- (n.=205); **b**: ortho-PO_4^{2-} (n. =136).*

NL= The Netherlands, BE.FL= Belgium-Flanders, L= Luxembourg, D= Germany, GB= United Kingdom, F= France, BE.WA= Belgium-Wallonia, LV= Latvia, IRL= Ireland, SLO= Slovakia, PL= Poland.

Although spring water PO_4^{2-} concentrations were much lower, a comparable pattern for NO_3^- was observed (Figure 2b). Highest PO_4^{2-} levels were again found in The Netherlands (average 1.07 µmol PO_4^{2-} l^{-1}) followed by Germany (average 0.57 µmol PO_4^{2-} l^{-1}). Other countries had less data or even no data on PO_4^{2-} concentrations but when present they were low (on average < 0.32 µmol PO_4^{2-} l^{-1}).

Chemical surface water composition and the occurrence of bryophytes

A redundancy analysis (RDA) was carried out including both bryophyte composition and the chemical water composition of all petrifying springs sampled to find (dis)similarities between both datasets (n=51, Figure 3).

The first axis of the RDA explains 28% and the second axis explains 14% of the variation in water composition (total 42%). For the variation in bryophyte composition the first axis of the RDA explains 19% and the second axis explains 7%.

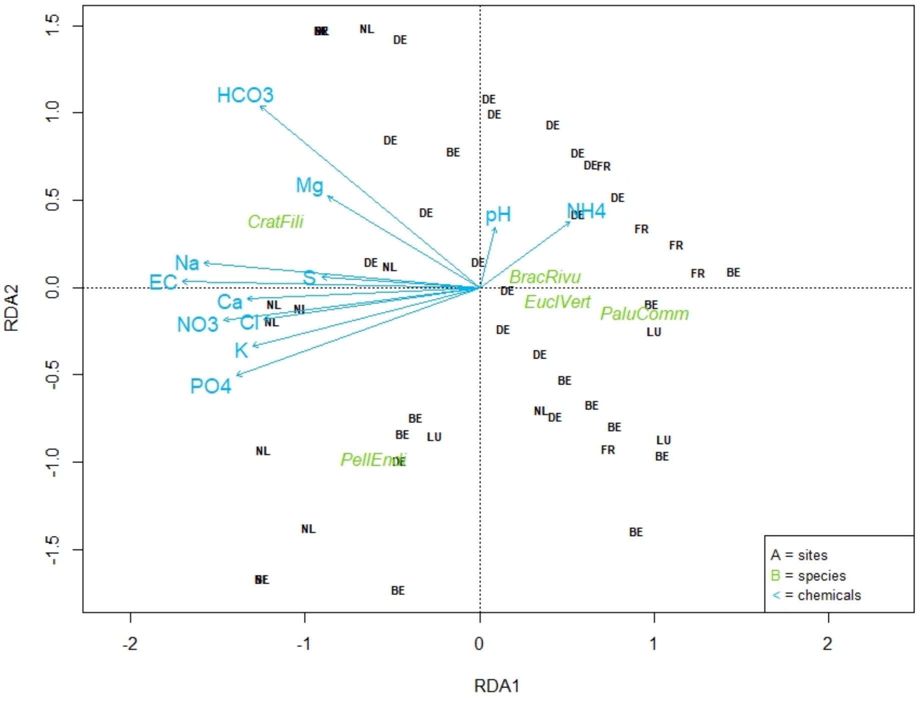

Figure 3: RDA diagram of the 51 petrifying springs sampled during this study.

Locations are presented in black, coded per country: NL = The Netherlands, BE = Belgium, LU = Luxembourg, DE = Germany, FR = France. The five petrifying moss species are presented in green and chemical parameters in blue. The length of the green arrows indicates the amount of the variance in bryophyte composition explained by the chemical parameter.

Several chemical parameters are rather strongly correlated (i.e. Ca^{2+}, Na^+, Cl^-, NO_3^- PO_4^{2-} and K^+ in the left side of the diagram; (Figure 3). Mg^{2+} and HCO_3^- show a somewhat different pattern and point to the upper left corner. NH_4^+ and pH pointing to the upper right corner of Figure 3, do not explain much of the variation. The occurrence of *Cratoneuron filicinum*, and to a lesser extent also *Pellia endiviifolia,* is associated with high nutrient concentrations and high concentrations of HCO_3, Ca^{2+}, and Na^+. However, *Palustriella commutata* and to a lesser extent *Eucladium verticillatum* and *Brachythecium rivulare,* show the opposite trend and occur at lower nutrient- and base cation concentrations. Most Dutch petrifying springs and some springs in Belgium and Germany are found at the left side of the RDA diagram corresponding to *Cratoneuron filicinum* dominated springs with high nutrient and base cations concentrations. In the lower left corner of the RDA diagram (Figure 3), a series of Belgian and some Dutch petrifying springs are associated with the presence of *Pellia endiviifolia*. Most of the petrifying springs dominated by *Palustriella commutata* originate from France, Luxembourg and partly from Germany and Wallonia. These sites are all associated with relatively low concentrations of nutrient and base cations.

The coverage of *Palustriella commutata* showed a significant negative correlation with spring water NO_3^- and PO_4^{2-} concentrations (Table 1). A high cover of *Cratoneuron filicinum,* however, showed significant positive correlations with high NO_3^- and PO_4^{2-} concentrations. The cover of the bryophytes *Eucladium verticillatum* and *Brachythecium rivulare* showed non-significant negative correlations with concentrations of NO_3^- and PO_4^{2-} (Table 1). The cover of *Pellia endiviifolia* showed a non-significant positive correlation with the groundwater NO_3^- concentrations but a significant positive correlation with PO_4^{2-} concentrations (Table 1).

Table 1: Linear correlation results of the correlation between spring water NO_3^- concentration and ortho- PO_4^{2-} concentration and the coverage of the five bryophyte species typical for petrifying springs.

	NO_3^-		**Ortho-PO_4^{2-}**	
	R^2	***P***	***R^2***	***P***
Cratoneurion filicinum	0.168	**0.003**	0.106	**0.020**
Palustriella commutata	-0.166	**0.003**	-0.121	**0.012**
Brachythecium rivulare	-0.064	0.073	-0.045	0.135
Eucladium verticillatum	-0.039	0.162	-0.038	0.172
Pellia endiviifolia	0.062	0.079	0.095	**0.028**

For NO_3^- the three quality categories (poor, moderate and good) are plotted in Figure 4a showing that locations with a poor (impoverished) quality occur over a broad range of NO_3^- concentrations, but most of these locations have a high NO_3^- concentration of more than 30 mg NO_3^- l^{-1}. Both the locations of moderate and good quality are characterised by significantly ($P < 0.005$) lower NO_3^- concentrations. The mean NO_3^- concentrations in the classes moderate and good do not, however, differ significantly from one another. The moderate category represents a group of petrifying springs already under stress. The 95th percentile of the moderate category is higher than for the good category and does show pronounced outliers, while the coverage of the critical bryophyte species is relatively low. According to the method used the threshold value (Figure 4a) for NO_3^- is found at 288 µmol NO_3^- l^{-1} (18 mg l^{-1}).

Threshold values for PO_4^{2-} concentrations in petrifying springs were calculated in a similar way as NO_3^- (Figure 4b). Also, for PO_4^{2-} significantly higher PO_4^{2-} concentrations were found in the poor-quality category ($P <0.005$) compared to the categories ‘moderate’ and ‘good’. The threshold value for PO_4^{2-} was determined at 0.42 µmol PO_4^{2-} l^{-1} (0.04 mg l^{-1}).

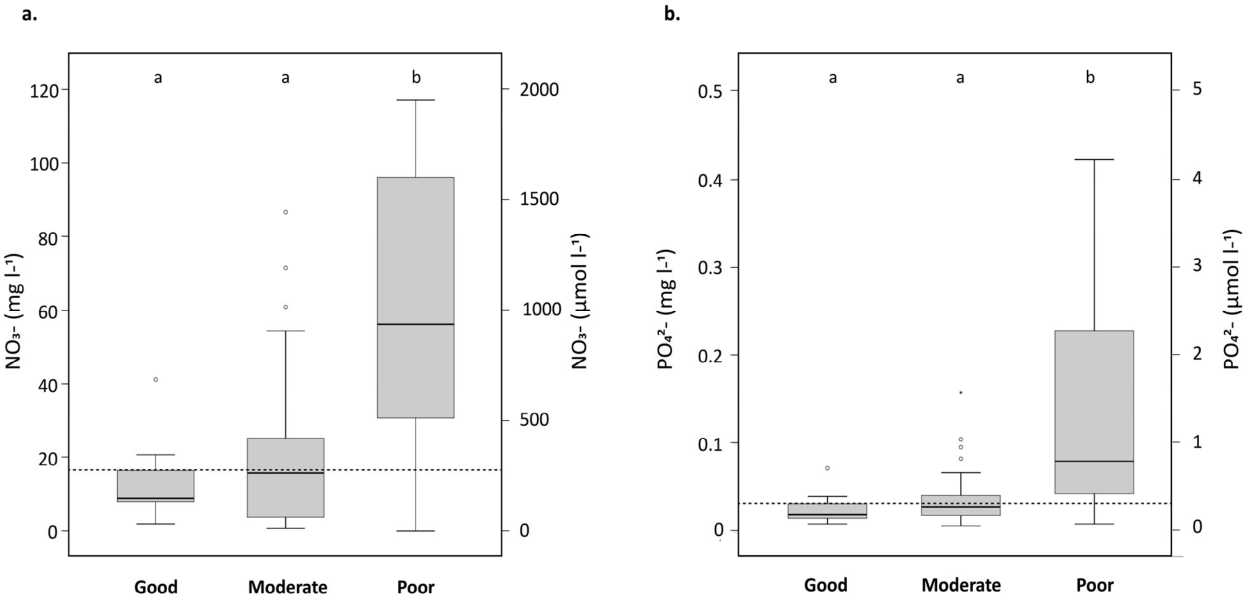

*Figure 4: Boxplots of **a**. spring water NO_3^- concentrations; **b**. spring water PO_4^{2-} concentrations for the three quality categories of petrifying springs.*

Dotted lines are the defined threshold values. With letters the significant difference ($P < 0.005$) between the categories are indicated.

DISCUSSION

Characteristic bryophytes and nutrient loads in groundwater

Based on a dataset with a broad range of unpolluted to heavily polluted springs in Europe clear trends and significant correlations were identified between the coverage of individual bryophyte species and groundwater nutrient concentrations. Groundwater NO_3^- concentrations in petrifying springs in The Netherlands are 5-10 times higher than in petrifying springs in Germany, southern Belgium and Eastern Europe. Compared to the high N loads associated with groundwater the direct influence of N deposition on petrifying springs is negligible (Bobbink & van Dijk, 2017). Additionally, PO_4^{2-} and Cl levels showed a comparable trend to NO_3^- levels which are related to fertilizer application and not to aerial deposition (Appelo & Postma, 2005). This indicates that nutrient leaching from agricultural fields is the dominant factor, especially in The Netherlands and Flanders. Groundwater nutrient pollution does affect the bryophyte species of petrifying springs but not all species are affected in the same way.

As far as NO_3^- is concerned our threshold value of 288 µmol NO_3^- l^{-1} for the habitat type 7220 is comparable to the more generally defined threshold value for lowland petrifying springs in the United Kingdom (320 µmol NO_3^- l^{-1}) previously determined by (UKTAG, 2012). On the other hand, various regional studies addressing the relationship between spring water pollution and species composition could not detect clear effects of nitrate or phosphate pollution on species composition (Hájková et al., 2012; Farr et al., 2014; Grootjans et al., 2015; Lyons, 2015; Couvreur et al., 2016; Farr & Graham, 2017). However, these studies involved predominantly sites with either very low pollution levels or rather polluted sites (Oosterlynck & De Bie, 2000). The limited range of pollutant concentrations in these studies may explain why these studies could not detect these relations.

When we focus on spring phosphate concentrations, Farr et al. (2014) did not find *Palustriella commutata* and *Eucladium verticillatum* in springs supplied by water exceeding 0,05 mg/l PO_4^{2-}. Our data showed both species to be mainly present in locations which we allocated to the 'moderate' and 'good' classes. The defined threshold values for these classes, ('good': 0,04 mg l^{-1}; 'moderate' 0,05 mg l^{-1}) are comparable to the findings of Farr et al. (2014).

The presence and abundancy of *Pellia endiviifolia* seems to be already stimulated at low levels of PO_4^{2-}. Oosterlynck & De Bie (2000) sampled mostly rather intermediate

NO_3^--polluted springs which were usually quite low in phosphate (Figure 2; Supplementary Fig. S2). Because of the high frequency of this species on their studied sites they ranked it as a characteristic species for petrifying springs in Flanders. Similar situations with enhanced phosphate availability are reported for slightly polluted streams in Ireland, the United Kingdom and Wallonia where *Pellia endiviifolia* is present on many tufa dams and barrages (Lyons, 2015; Bouxin et al., 2016; Farr & Graham, 2017). In The Netherlands this species is not exceptionally frequent in petrifying springs. Even though the landscape ecological setting of these springs is not very different to Flanders. However, in The Netherlands the concentrations of both nitrate and phosphate in spring waters are much higher (Figure 2). It is possible that in such conditions the presence and abundancy of *Pellia endiviifolia* is reduced again. In contrast to this, (Couvreur et al., 2016) detected a negative correlation to P-availability but remarkably also a positive correlation with nitrate which could not be explained. A possible explanation could be that within their relatively small study area (Wallonia) the range of nitrate concentrations in springs and brooks is often small compared to Flanders and The Netherlands (Figure 2a). Therefore the pollution gradient in the dataset might be too narrow to enable correlation with nitrate concentrations and habitat condition

We conclude that bryophyte species listed as characteristic species for the habitat type 7220 “Petrifying springs with travertine formation (Cratoneurion)” react very differently NO_3^- concentrations. Species abundancy of *Palustriella commutata* and to a lesser extent *Eucladium verticillatum* and *Brachythecium rivulare* showed negative correlations with increasing spring water nutrient concerntrations. In contrast, the abundance of *Cratoneuron filicinum* and to a lesser extent *Pellia endiviifolia* showed positive correlations with increasing nutrient concerntrations in spring water. Hence, we conclude that *Palustriella commutata, Eucladium verticillatum* and to a lesser extent *Brachythecium rivulare* have a strong preference for unpolluted petrifying springs, whereas *Cratoneuron filicinum* and *Pellia endiviifolia* show a broader tolerance to water pollution. Of these two species *Cratoneuron filicinum* is certainly not the best indicator of “good health” of the habitat type 7220 “Petrifying springs with travertine formation (Cratoneurion)”, especially when it is the only species present as often found in low quality nutrient enriched forms of this habitat.

Groundwater composition under NO_3^- enriched conditions

Nutrient pollution may not only affect N and P availability for bryophytes but may also affect the chemical composition of the ground and spring water. The sum of Ca^{2+} and Mg^{2+} also showed a significant correlation with NO_3^- concentrations. The highest Ca^{2+}+Mg^{2+} and HCO_3^- concentrations are also found in these, mainly (sandy) loess and loamy areas and not in the typical chalk districts like the Condroz (B), Plateau de Langres (F), the Kalk-Eifel and Saargau-Merzig (D) as might be expected (Figure 1). This suggests interference of NO_3^- with calcite ($CaCO_3$) and dolomite ($CaMgCO_3$) dissolution processes, particular in the loess and loamy areas (Appelo & Postma, 2005).

A further analysis of our dataset (Figure 5A) revealed that for a subset of the studied sites Ca^{2+}+Mg^{2+} and HCO_3^- concentrations showed an exact 1:2 ratio which means that for these waters Ca^{2+} (Mg^{2+}) carbonate dissolution by carbonic acid (infiltrating rain water) is likely to be the main process determining Ca^{2+}+Mg^{2+} and HCO_3^- concentrations in the spring waters conform Equation (1).

Eq. (1): $CaCO_3 + H_2CO_3 \rightarrow Ca^{2+} + 2\,HCO_3^-$

These locations showed hardly any evidence of pollution (NO_3^- <100 µmol l^{-1}). For the remaining sites, however, the ratio between HCO_3^- and Ca^{2+}+Mg^{2+} levels are much lower than 2 (Figure 5a), including almost all locations in The Netherlands and the Northern part of Belgium (Flanders). This also indicates that other processes contribute to the dissolution of Ca^{2+} and Mg^{2+}.

NO_3^- leaching, especially in regions with intensive agiculture for example The Netherlands, must be pinpointed as the main source of nitric and sulphuric acids in the groundwater (Van Diggelen et al., 1995; Goodchild, 1998; Iversen et al., 1998; Kirchmann et al., 2002). Eventually several buffering reactions (consumption of alkalinity, dissolution of carbonates and cation exchange reactions) result in the release of Ca^{2+}+Mg^{2+} and a net release of HCO_3^- which explains why Ca^{2+}+Mg^{2+} also show a correlation with NO_3^- in spring waters in areas which are impacted by the leaching of NO_3^-.

Apart from the processes mentioned above Ca^{2+} and Mg^{2+} may also be provided by the application of artificial fertilizers such as $Ca(NO_3)_2$ or $CaNH_4NO_3$. The latter calcium-ammonium-nitrate (CAN) is the most used N-fertiliser in the European Union, especially in North-Western Europe (Sutton et al., 2011). CAN fertlisers are often added to counteract acidification of the soil and to replenish the soil, Mg^{2+}. Excessive amounts of NO_3^- provide a negative counter charge, which also promotes concurrent leaching of these cations (Di & Cameron, 2002). After leaching through the soil, they will enter the groundwater system and discharge via springs with enhanced concentrations of these cations being an indication of excessive use of artificial fertilisers.

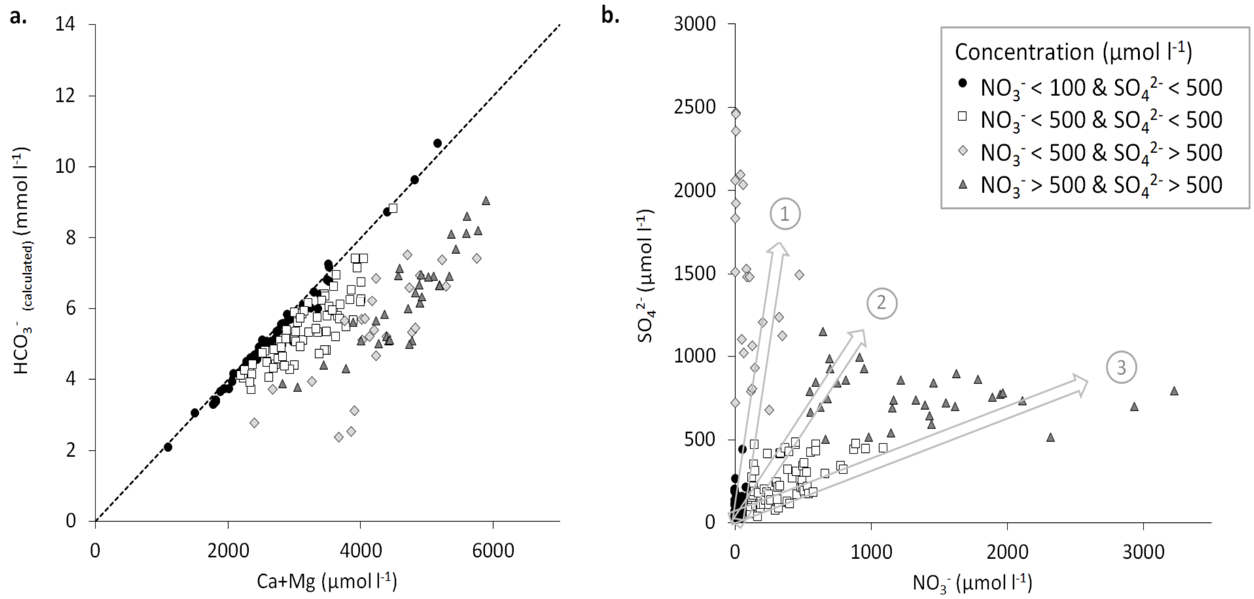

*Figure 5: **a**. Relation between Ca^{2+}+Mg^{2+} and calculated HCO_3^- concentration in spring water of 183 petrifying springs. **b**. Relation between the NO_3^- concentration and the SO_4^{2-} concentration. The samples have each been assigned to one of four groups according to their NO_3^- and SO_4^{2-}- concentrations.*

In the petrifying springs dataset, three groups can be identified outside of the unpolluted locations (Figure 5a, b).defined by differences in their relative concentrations of NO_3^-- and SO_4^{2-}. In these three groups we can recognize different pathways of NO_3^- reduction. The pathways are possibly related to the hydrogeological reductive capacities of the supporting aquifers, which are determined by the presence of sedimentary organic matter (SOM) and reduced iron and sulphur bearing deposits (Straub et al., 1996; Moncaster et al., 2000; Appelo & Postma, 2005; Haaijer et al., 2007; Burgin & Hamilton, 2008).

- *Group 1* reflects two different processes - (i) dissolution of gypsum, which in the chalk districts is often a natural constituent of the geological deposits. - (ii) leaching and reduction of nitrates (high sulphate, low NO_3^-). The oxidation of subsoil iron-sulphides by NO_3^- prevents the accumulation of NO_3^- in groundwater but results in high sulphate concentrations (Smolders et al., 2010; Van Dijk et al., 2019). Locations in this second group affected by this process are usually located outside the chalk districts.
- *Group 2:* , reflects oxidation of iron-sulphides and iron-carbonates by NO_3^- in the subsoil (Goossens, 2007) which is the main pathway for NO_3^- reduction, however it cannot prevent NO_3^- leaching to the groundwater entirely. Most of the Flemish samples belong in this group.

- *Group 3*: Represents NO_3^- reduction due to the oxidation of iron-sulphides. This process may be slow as there is only a limited increase of the sulphate concentrations with strongly increasing nitrate concentrations. This indicates that the availability of sulphur bearing deposits is limited while also the alternative, SOM, is often absent in the subsoil (Appelo & Postma, 2005), so most NO_3^- ends up in the groundwater. The majority of Dutch samples and some Flemish samples are found in this group.

Apart from the above-mentioned processes the application of $(NH_4)_2SO_4$ fertilizers, often used on alkaline soils, may also contribute to an increased leaching of sulphate to the groundwater.

Intensive fertilisation can cause significant changes in groundwater chemistry with consequences for petrifying spring habitats. Apart from the substantial base cation enrichment the polluting effect of NO_3^- and SO_4^{2-} on spring water chemistry may differ in each region depending on the specific regional hydrogeological setting.

Although the present field study shows clear correlations between groundwater nutrient concentrations and bryophyte cover, we cannot draw conclusions about how groundwater nutrient enrichment exactly affects the physiological and ecological functioning of bryophyte species listed for the habitat type 7220. Furthermore, we do not know how other differences in ground water chemistry, resulting from the excessive leaching of NO_3^- may affect the physiological and ecological functioning of petrifying spring specific bryophyte species. Further research should focus on the physiological and ecological effects of, for instance, artificially increased Ca^{2+}+Mg^{2+} and/or NO_3^- concentrations in spring water.

There may be also another potential problem which deserves further research. Laboratory experiments have shown that with increasing PO_4^{2-} concentrations the petrifying process is increasingly reduced. At concentrations of 1 - 3 μmol PO_4^{2-} l^{-1}, as is regularly encountered in springs in The Netherlands (Figure 2b), the petrifying process could be limited by 30 - 80% (Tadier et al., 2017; Zeppenfeld, 2019). The existing travertine becomes more enriched with phosphates by the increasing absorption of $CaHPO_4$-species, which may alter the site conditions for mosses. Both processes could have a severe impact on the basic characteristics and development of the petrifying spring habitat.

Implications for Nature management and Environmental policy

In this study we found strong indications that petrifyuing springs can be impacted by enhanced groundwater nutrient concentrations derived from high NO_3^- leaching in the catchment area. In The Netherlands and Flanders especially, nutrient leaching from agricultural fields appears to be the dominant factor. Direct influence of atmospheric N deposition on petrifying springs is limited, compared to the high groundwater N loads (Bobbink & van Dijk, 2017).

In order to preserve and restore endangered and sensitive petrifying spring habitatsmeasures should be taken to prevent the very high nutrient losses associated with application of artificial fertilizers. Within the groundwater recharge areas of vulnerable petrifying spring habitats support to farmers should be provided to reduce the application of fertilisers or to change to sustainable practises to prevent excessive nutrient loading via local or regional hydrological systems. A sharp reduction of nutrient leaching (nitrate & phosphate) will contribute to the restoration of a more natural nutrient poor type of spring water chemistry and will also supress excessive NO_3^- induced cation and sulphate leaching.

It is important to state that the defined threshold value for NO_3^- of 18 mg l^{-1} is significantly below the NO_3^- drinking water standard of the European Nitrates Directive of 50 mg/l NO_3^- (806 µmol NO_3^- l^{-1}), although in several countries such as The Netherlands even this standard is still not met (Figure 2a; Van Grinsven et al., 2012).

This stresses the necessity of further and stronger regulation for both NO_3^- and PO_4^{2-} pollution of groundwater in Europe.

CONCLUSIONS

Groundwater composition and bryophyte composition in 51 NW European petrifying springs indicates that severe groundwater nutrient enrichment (NO_3^- and PO_4^{2-}) has potentially negative consequences for the occurrence of bryophyte species characteristic of petrifying springs. Bryophyte species abundance of *Palustriella commutata* and to a lesser extent *Eucladium verticillatum* and *Brachythecium rivulare* showed negative correlations with increasing nutrient and base cation levels in the groundwater. However, the abundancy of *Cratoneuron filicinum* and to a lesser extent *Pellia endiviifolia* showed positive correlations with increasing nutrient and base cation levels. On this basis, our initial hypothesis is only partly correct. Because of the positive response to NO_3^- *Cratoneuron filicinum* should no longer be labelled as a species associated with high quality petrifying springs habitat.

In watersheds with intensive agriculture, the most plausible source for nutrient enrichment is nutrient leaching from nearby intensively fertilized agricultural fields while forests influenced by atmospheric N deposition may also play a role. We were able to clarify that NO_3^- leaching influences biogeochemistry in the aquifer which inevitably results in an alteration to water chemistry, as we hypothesized, not only loaded with nutrients but also in increased base cation concentrations and often SO_4^{2-}. The effect of high concerntrations of NO_3^- on spring water chemistry may, however, differ considerably between regions depending on the specific hydrogeological reductive capacities of the regional aquifers feeding the petrifying springs. This may also result in ecologically different responses to pollution between regions. Conservation and restoration strategies must aim to reduce the application of fertilisers to prevent exessive nutrient leaching to groundwater.

Based on the present study we propose threshold values of 288 µmol NO_3^- (18 mg l^{-1}) and 0.42 µmol PO_4^{2-} l^{-1} (0.04 mg l^{-1}) to maintain favourable ecohydrological conditions for development and sustainability of petrifying springs habitat and their associated characteristic bryophyte species.

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Supplementary Table S1

Number of samples used in the present analysis obtained from own research and derived from literature.

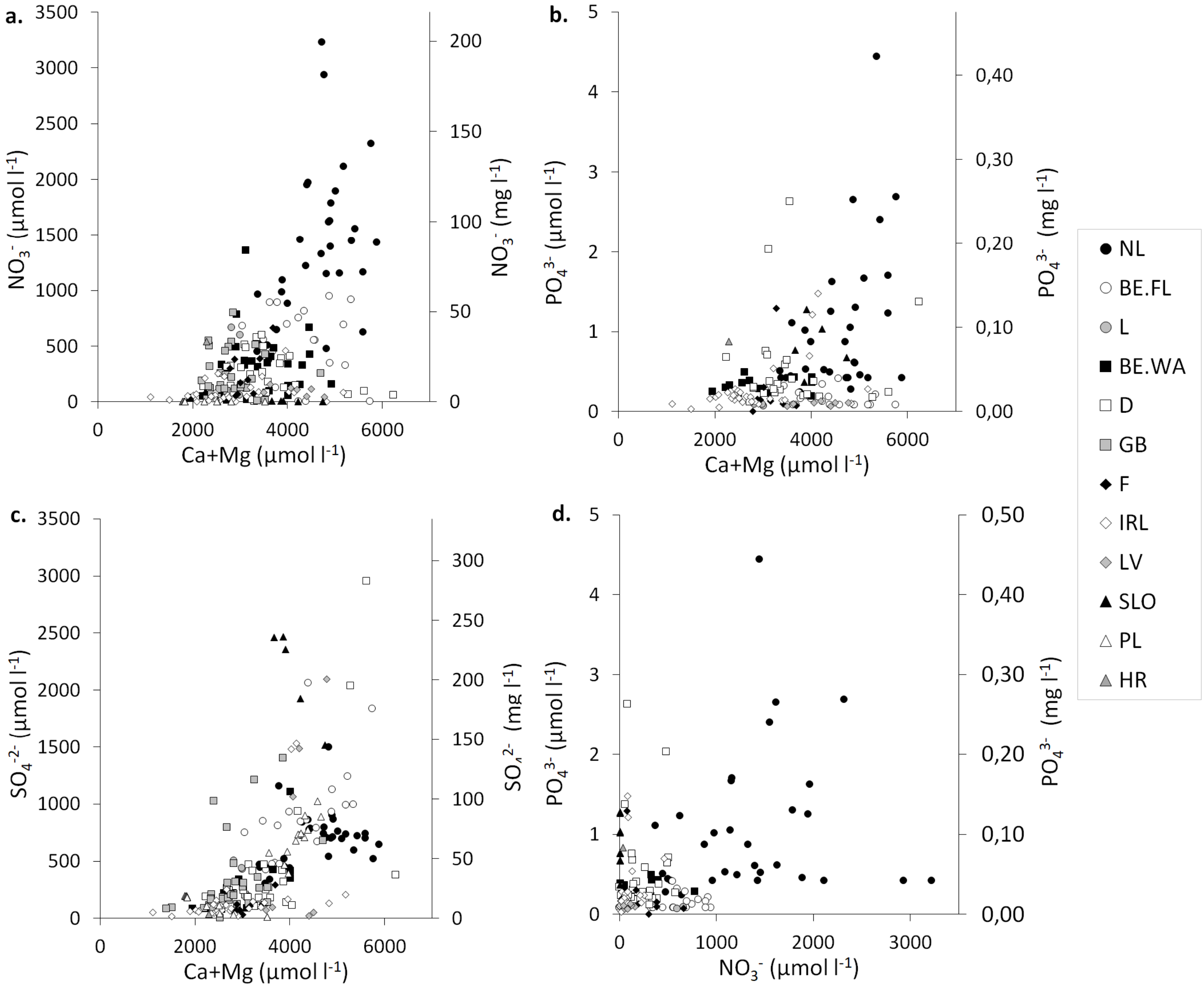
Country	**Samples present study**	**Additional samples**	***References***
Belgium – Flanders	2	20	Oosterlynck & De Bie, 2000
Belgium – Wallonia	10	16	Couvreur et al., 2016;
Croatia		1	De Mars et al., 2016
France	5	4	Hoffman, 2005; De Mars unpubl., 2019
Germany	20	5	Arp et al., 2010; Hartkopf-Froder et al., 1989
Ireland		32	Frisia et al., 2000; Lyons, 2015
Latvia		7	Grootjans et al., 2015
Luxembourg	3		
Poland		24	Grootjans et al., 2015
Slovakia		10	Hájková et al., 2012
The Netherlands	11	20	Database Waterboard Limburg, 2015; Database BWARE, 2015
The United Kingdom		34	Pentecost & Zhaohui, 2002; Columbu et al., 2013; Thomas, 2007; Farr et al., 2014; Farr & Graham, 2017;
Total	**51**	**173**	

Supplementary Figure S2

Four scatter plots for the complete dataset (locations samples within this study and data from literature, when data was available for presented parameters.

a*: The* Ca^{2+}+Mg^{2+} *concentration in relation to the* NO_3^-*concentration.* ***b****: The* Ca^{2+}+Mg^{2+} *concentration in relation to ortho-* PO_4^{2-} *concentration.* ***c****: The* Ca^{2+}+Mg^{2+} *concentration in relation to the* SO_4^{2--} *concentration.* ***d****: The* NO_3^- *concentration in relation to the* PO_4^{2-} *concentration.*

NL= The Netherlands, BE.FL= Belgium, Flanders, L= Luxembourg, BE.WA = Belgium, Wallonia, D= Germany, GB= United Kingdom, F= France, , IRL= Ireland, LV= Latvia, SLO= Slovakia, PL= Poland, HR = Croatia.



Supplementary Table S3

Overview of the water chemistry of 224 selected European petrifying springs, including location specifications and additional information.

(separate)

***Supplementary Figure S4*:**

Species cover response to NO_3^-

(separate)