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Interactions between pH and nutrients on benthic algae in streams and consequences for ecological status assessment and species richness patterns

Susanne C. Schneider ^{1*}, Maria Kahlert ², Martyn G. Kelly ³

1 Norwegian Institute for Water Research, Gaustadalleen 21, 0349 Oslo, Norway;
susi.schneider@niva.no; +47 98294098

2 Swedish University of Agricultural Sciences, Dept. of Aquatic Sciences and Assessment,
PO Box 7050, SE-750 07 Uppsala, maria.kahlert@slu.se , +46 18-67 31 45

3 Bowburn Consultancy, 11 Montaigne Drive, Bowburn, Durham DH6 5QB, UK;
MGKelly@bowburn-consultancy.co.uk ; +44 191 377 2077

* Corresponding author

Abstract

Eutrophication and acidification are among the major stressors on freshwater ecosystems in northern Europe and North America, but possible consequences of interactions between pH and nutrients on ecological status assessment and species richness patterns have not previously been assessed. Using data from 52 river sites throughout Norway, we investigated the combined effects of pH and nutrients on benthic algae assemblages, specifically 1) taxa-specific couplings between nutrient and acidity traits, 2) the degree of consistency between different biotic indices, separately for nutrients and acid conditions, 3) the impact of pH on nutrient indices and phosphorus on indices of acid conditions, and 4) the impact of pH and phosphorus supply on diatom and non-diatom taxon richness. We found that 1) acid-tolerant taxa are generally associated with nutrient-poor conditions, with only a few exceptions; this is probably more a consequence of habitat availability than reflecting true ecological niches; 2) correlation coefficients between nutrient indices and TP, as well as acid conditions indices and pH were barely affected when the confounding factor was removed; 3) the association of acid-tolerant taxa with nutrient-poor conditions means that the lowest possible nutrient index at a site, as indicated by benthic algae, is lower at acid than at circumneutral sites. Although this may be an

artefact of the datasets from which taxa-specific indicator values were derived, it could lead to a drift in nutrient indices with recovery from acidification; 4) the response of non-diatom taxon richness follows a complex pattern with a synergistic interaction between nutrient supply and pH. In contrast, diatom richness follows a simple additive pattern; this suggests structural differences between diatoms and non-diatom benthic algae in their response to nutrient supply and pH; diatom taxon richness tended to increase with nutrient supply, while non-diatom richness decreased.

Keywords

Phosphorus, acidity, periphyton, multiple stressors, eutrophication, acidification

1. Introduction

Freshwater ecosystems have long been affected by various types of human impact, with negative consequences on water quality and biota (Søndergaard & Jeppesen, 2007). Eutrophication, manifested in excessive growth of algae and submerged macrophytes, is one of the most important pollution problems in lakes and rivers in the developed world (Hilton et al., 2006). Although improved waste water treatment, reduction of external nutrient loading, and restoration measures have reduced nutrient concentrations in many freshwater ecosystems, eutrophication still is a major impact factor in many countries (Søndergaard & Jeppesen, 2007). Acidification, with its associated decline in salmon and trout populations, has also been a key factor in some areas, including large parts of Scandinavia (Skjelkvåle et al., 2005) and North America (Clair et al., 2011). Although Scandinavian surface waters have been recovering from acidification since the early 1990s in response to lower levels of acid deposition (Skjelkvåle et al., 2005), acid precipitation continues to exceed the critical load of many surface waters in sensitive areas such as southern Norway (Wright et al., 2005). Since forestry can contribute to acidification by leading to a net loss of base cations as a consequence of whole-tree harvesting (Akselsson et al., 2007), and maritime influence and catchment features also are important, there is not necessarily a recovery in pH associated with declines in acid precipitation (Löfgren et al. 2009, 2011). Thus, both eutrophication and acidification will continue to be among the major impacts on freshwater ecosystems in the coming decades.

Limnologists have been aware of an interaction between pH and trophic status in some types of water bodies for about a century. Naumann (1929) described the difference between acidotrophic (low phytoplankton production, low macrophyte biomass, low pH) and alkalitrophic (low phytoplankton production, high macrophyte biomass, high Ca-concentration) lake types whilst Ohle (1955) mentioned that low pH can cause oligotrophic conditions in lakes. Acid precipitation, however, leads to depletion of soil base cation reserves eventually reaching a point where further acid buffering by base cations leads to the release of phosphates associated with these sites and, consequently, to eutrophication (Oxley & Allen, 2000). In addition, liming is commonly applied in many acidified areas in Scandinavia (see e.g. DN, 2010), and cessation of liming can lead to sudden release of phosphates from the soil into river systems (Oxley, 2000). Furthermore, field evidence in Scandinavia shows that both settlements and agriculture exist in areas affected by acidification. Freshwater ecosystems might therefore simultaneously be exposed to both, eutrophication and acidification pressures.

The Water Framework Directive (WFD: European Union, 2000) requires member states of the European Union to regularly assess, amongst other properties, phytobenthos in rivers. Though most use diatoms as proxies for the complete phytobenthos assemblage, the validity of this approach has been questioned (Schneider et al., 2012). Indices based on species-composition of non-diatom benthic algae have been developed for trophic status (periphyton index of trophic status PIT, Schneider & Lindstrøm, 2011) and acid conditions (acidification index periphyton AIP, Schneider & Lindstrøm, 2009) for rivers in Norway. Likewise, diatom based indices for nutrients/general pollution (Indice de Polluo-sensibilité Spécifique IPS (Coste in Cemagref, 1982) and acid conditions (ACID, Andrén & Jarlman, 2008) are used in Swedish rivers, while in the UK, the Trophic Diatom Index (TDI, Kelly et al., 2008) and the Diatom Acidification Metric (DAM, Juggins & Kelly, 2012) are used. As these countries are now assessing the status of nutrients and acid conditions from a single benthic algae sample it is important to understand how responses to nutrients and acid conditions are coupled, and the possible consequences for ecological status assessment (see e.g. Denys, 2004). Additionally, we expect that interactions between acid and nutrient conditions, if these exist, may conceal patterns in stressor – biodiversity relationships. This is important because ecological status in the WFD is defined via the structure and function of ecosystems.

In this study, we use data from 52 river sites throughout Norway to investigate the combined effects of pH and phosphorus supply on river benthic algae. Based on this dataset, which includes data on water chemistry, as well as diatom and non-diatom benthic algae species

composition, we analyse 1) taxa-specific couplings between nutrient and acidity traits, 2) the degree of consistency between biotic indices from Norway, Sweden and UK, separately for nutrients and acid conditions, 3) the impact of pH on nutrient indices and phosphorus on acid conditions indices, and 4) the impact of pH and phosphorus supply on diatom and non-diatom taxon richness. Our hypothesis is that co-variation between pH and nutrient concentrations interferes with both nutrient indices and taxon richness patterns.

2. Material and Methods

2.1 Dataset

Samples were taken at 52 river sites in Norway (Fig. 1). All river types (defined via calcium and total organic carbon concentration) and all ecoregions in Norway are represented, and the dataset spans both unimpacted “reference” sites and sites impacted by eutrophication and acidification (Table 1; see Schneider (2011) for a description of river types, ecoregions and selection of reference sites). Samples were collected during various projects between 1981 and 2007. Water chemistry samples were taken at the sampling sites between one and 24 times per year and the results stored in the database of the Norwegian Institute for Water Research (NIVA). TP was measured at 49 and pH at 46 sites, respectively, such that the complete dataset used for modelling taxon richness patterns included 43 sites. Water chemistry was analysed according to Norwegian standard (NS) procedures during all years (pH: NS 4720; total phosphorus (TP): NS 4725). Site-specific, mean-annual water chemistry data for the one year previous to the benthic algae sampling were used to characterize pH and TP concentrations. Average water chemistry data were used because i) the benthic algae indices we here analyse also were calibrated on average water chemistry data, ii) average chemistry often correlates better with biological response than extreme values (see e.g. Andrén & Jarlman, 2008), and iii) the nature of the chemistry database used prevents analysing anything else except the average. The terms “pH” and “acid conditions” are used throughout this paper to summarise all the characteristics (e.g. acid neutralising capacity, concentration of labile aluminium) that exert ecophysiological stresses on the aquatic biota.

2.2 sampling methods

Benthic algae, i.e. algae that live attached to the river bottom or in close contact on or within patches of attached aquatic plants, were surveyed once at each site during summer/autumn according to European standard procedures (EN 15708:2009) along an approximately 10-m length of river bottom using an aquascope. At each site, percent cover was noted for each form of macroscopically visible benthic algae, and samples were collected and stored separately in vials for species determination. In addition, diatoms and other microscopic algae were collected from ten cobbles and small boulders with diameters ranging between approximately 10 and 20 cm, taken from each site. An area of about 8 x 8 cm from the upper side of each stone was brushed with a toothbrush to transfer the algae into a beaker containing approximately 1 L of river water and a subsample was taken. All samples were preserved with a few drops of formaldehyde to a final concentration of approximately 0.5%. The preserved benthic algae samples were later examined under a microscope (200 to 600 × magnification) and all non-diatom algae identified to species level, wherever possible. The primary identification keys used were Geitler (1932), Komarek & Anagnostidis (2007), Gutowski & Förster (2009), and John et al. (2011) as well as the respective earlier editions of each of these. For each taxon of non-diatom benthic algae, percent cover at each site is stored in our database, but for this analysis, we only use presence-absence data.

Diatom sampling, treatment, identification and enumeration followed EN 13946:2006 and EN 14407:2007. Diatom samples were digested using a mixture of hydrogen peroxide and potassium hypochlorate. Permanent slides were prepared from the cleaned suspensions using Naphrax (refractive index = 1.74, Brunel Microscopes Ltd) as a mountant. 422 ± 8 (average \pm standard deviation) undamaged valves of non-planktonic taxa were identified and counted using 1000 × magnification. The primary floras and identification guides used were Krammer & Lange-Bertalot (1986-91), Lange-Bertalot (1993, 2001), Lange-Bertalot & Moser (1994), Lange-Bertalot & Metzeltin (1996), and Krammer (2000) with nomenclature updated to reflect current practices.

2.3 Indices

We calculated the non-diatom indices for trophic status (PIT) and acid conditions (AIP) applied in Norway (Schneider & Lindstrøm, 2009; 2011), the diatom acid conditions indices applied in Sweden (ACID; Andrén & Jarlman, 2008) and the UK (DAM; Juggins & Kelly, 2012), as well as the three most widely applied diatom indices for nutrient concentration/general pollution in

Europe, i.e. the IPS (Coste in Cemagref, 1982), TDI (Kelly et al., 2008), and TI (Rott et al., 1999).

PIT and AIP indices are calculated by averaging indicator values of the taxa present at a site. PIT ranges from 1.87 to 68.91, and low values indicate low phosphorus concentrations, while high values indicate high phosphorus concentrations. To calculate a reliable PIT, at least two indicator taxa need to be present at a sampling site. The AIP index ranges from 5.13 to 7.50, and low values indicate acid conditions, while high values indicate neutral to slightly alkaline conditions. To calculate a reliable AIP index, at least three indicator taxa need to be present at a sampling site (see Schneider & Lindstrøm, 2009; 2011 for details on PIT and AIP indices and reliability). Only reliable PIT and AIP indices (52 for PIT, 47 for AIP, respectively) were used in our study.

The IPS, TDI, TI and DAM are all based on the weighted average equation of Zelinka & Marvan (1961); the IPS was developed to reflect general pollution gradients but is usually strongly correlated with both nutrients and organic pollution whilst TI and TDI focus on the response of diatoms to inorganic nutrients. The IPS indicator values for individual taxa range from 1 to 5, whilst the calculated IPS index for a site ranges from 1 to 20, with low numbers indicating 'very heavy pollution', and high values indicating 'nutrient poor to very nutrient poor and no pollution' (Coste in Cemagref, 1982). For the sake of linguistic simplicity, we will hereafter refer to the IPS as a nutrient index. Indicator values for the TI and TDI range from 1 to 4 and 1 to 5 respectively, with low values indicating a preference for nutrient poor conditions, and high values indicating a preference for nutrient-rich conditions (see Rott et al., 1999 and Kelly et al., 2008 for details on TI and TDI).

DAM and ACID measure the response of diatoms to pH. ACID is the sum of two parts: the ratio between the circumneutral *Achnantheidium minutissimum* (Kützing) Czarnecki *sensu lato* and the acidophilic genus *Eunotia* Ehrenberg, and the ratio between the sum of all circumneutral, alkaliphilous and alkalibiont taxa to the sum of all acidophilic and acidobiont taxa (based on van Dam et al., 1994 and the subsequent updates in OMNIDIA, Lecoq et al., 1993). ACID indicator values for individual taxa range from 1 to 5, whilst the ACID calculated for a site ranges from 1 to 10, with low values indicating acid conditions. ACID is correlated to the mean pH of the year before diatom sampling (see Andrén & Jarlman, 2008 for details on ACID). DAM also divides diatoms into classes based on their sensitivity to pH but is calculated

using a weighted average equation. DAM indicator values for individual taxa range from 1 to 5, with low values indicating acid conditions (see Juggins & Kelly, 2012 for details on DAM).

To provide a link to ecosystem structure, we additionally calculated diatom and non-diatom taxon richness, as well as diatom genus richness for each sample. We refer to “taxa” rather than “species” to recognize that entities identifiable with the light microscope and current literature may not always reflect true biological species. Assignment of diatoms and non-diatoms into taxa was primarily based on the determination guides cited above. For some genera of filamentous green algae whose vegetative forms cannot be determined to species level (e.g. *Spirogyra* Link or *Mougeotia* C.Agardh) categories which are based mainly on filament width were used (see Schneider & Lindstrøm (2009; 2011) for further details). The same taxonomic levels were used consistently for analysis of all sites in our study.

2.4 Data treatment

TP-data were log-transformed to achieve normality. Visual inspection of the data clearly showed the absence of sites with both low pH and high TP concentrations (Fig. 2). We studied the consequences of this skewness on benthic algae indices and univariate taxon richness patterns by comparing results for the full dataset with datasets where the skewness was removed. To investigate the influence of pH, we used only sites with $TP < 16\mu\text{g L}^{-1}$ (37 sites; hereafter referred to as “acidity only” subset). For this subset, there is no correlation between $\log_{10}(TP)$ and pH (Pearson correlation coefficient -0.04, $p = 0.84$), while the length of the gradient in pH is retained (Fig. 2). To investigate the influence of TP, we used only sites with $pH > 6.5$ (27 sites; hereafter referred to as “nutrients only” subset). Again, for these sites, there is no correlation between $\log_{10}(TP)$ and pH (Pearson correlation coefficient -0.27, $p = 0.17$), while the gradient length in TP is retained (Fig. 2).

Spearman correlation was used to test for correlations among indices, and between indices and water chemical parameters, because we expected the correlations to be monotonic, but not necessarily linear. Because each analysis represented a separate hypothesis, there was no need to adjust α for multiple testing (Perneger, 1998). All tests were performed with STATISTICA 10.

We used quantile regression to investigate the highest and lowest values (as indicated by 95th and 5th percentiles) attained by TP-concentrations and nutrient indices at different acid conditions (as indicated by pH and acid condition indices), because (a) quantiles are less affected by extreme observations, and (b) quantile regression enables estimations of the minimum and maximum response. Quantile regression was done using the “quantreg” package (Koenker, 2010) in R version 2.14.2 (R Development Core Team, 2012).

To explore the interaction between pH and nutrient supply on taxon richness patterns, we used a set of multivariate linear regression models and selected the best model by using an information-theoretic approach. For this purpose, data were centered and standardized to 2 SD, following the recommendations of Quinn & Keough (2002) and Schielzeth (2010). Centering and scaling allows using the regression coefficients as measures of effect size (Schielzeth, 2010; see also Wagenhoff et al. (2011), who adopted a similar approach). We fitted our data to a multivariate second-order polynomial function of the form:

$$\text{richness} = b_0 + b_1 * \text{pH} + b_2 * (\text{pH})^2 + b_3 * \log\text{TP} + b_4 * (\log\text{TP})^2 + b_5 * \text{pH} * \log\text{TP}$$

Based on this model (called the “full model”), we used the dredge-procedure in R extended with the MuMIn package (Barton, 2012) to select the model best describing richness based on the lowest value of AIC_c (Akaike information criterion for small samples; Burnham & Anderson, 2002). When a polynomial term was retained, then the 1st order term was retained as well. We report the standardized regression coefficients plus 95% CIs of the top model (in case of non-diatoms) or of the averaged models (in case of diatoms). For diatom taxon and genus richness, averaging of the top 2 AIC_c models was applied, since they had only slightly different Akaike weights (Burnham & Anderson, 2002). An inclusion of zero in the 95% CI of the regression coefficients means that the effect is not statistically significant at $\alpha = 0.05$ (Nakagawa & Cuthill, 2007).

3. Results

At the 52 sites investigated, we found a total of 178 taxa of non-diatom benthic algae, the majority belonging to the classical green algae (Viridiplantae; 79 taxa) and cyanobacteria (76 taxa). The most frequent taxa were “*Mougeotia a*” (a narrow *Mougeotia* taxon with a filament width between 6 and 12 μm ; found at 28 sites), *Cosmarium* sp. Corda ex Ralfs (24 sites),

Closterium sp. Nitzsch ex Ralfs (18 sites) and *Binuclearia tectorum* (Kützing) Berger ex Wichmann (18 sites). We also found a total of 308 diatom taxa belonging to 28 genera; the most frequent were *Tabellaria flocculosa* (Roth) Kützing (occurring at 49 sites), *Achnantheidium minutissimum* (Kützing) Czarnecki (42) *sensu lato*, and *Fragilaria gracilis* Oestrup (30). A total of 100 diatom, and 64 non-diatom taxa were only recorded once. All nutrient indices were correlated to TP, and all acid conditions indices were correlated to pH, indicating that both diatom and non-diatom indices reflected well the pressure they were expected to indicate (Tables 2 and 3).

3.1 Species specific nutrient and pH indicator values

In total, 77 non-diatom taxa have both PIT and AIP indicator values and consequently have rather narrow ecological amplitudes with respect to both pH and TP. With the exception of *Microspora abbreviata* (Rabenhorst) Lagerheim, all taxa indicative of nutrient rich conditions with a PIT ≥ 10 have an AIP index above 7 (Fig. 3a), corresponding to a pH optimum above 7 (Schneider & Lindstrøm, 2009). Prominent taxa indicative of acidic and nutrient-poor conditions (bottom left hand corner of Fig. 3a) are *Batrachospermum keratophyllum* Bory de Saint-Vincent, *Hapalosiphon hibernicus* West & G.S.West, *Capsosira brebissonii* Kützing ex Bornet & Flahault, *Scytonematopsis starmachii* Koválik & Komárek, *Binuclearia tectorum* (Kützing) Berger ex Wichmann, narrow *Mougeotia* C.Agardh taxa (10-18 μm) and several *Stigonema* C.Agardh ex Bornet & Flahault species. Prominent taxa indicative of circumneutral and nutrient-poor conditions (bottom right hand corner of Fig. 3a) are *Hydrurus foetidus* (Villars) Trevisan, *Tolypothrix distorta* var. *penicillata* (C.Agardh) Lemmermann, broad *Mougeotia* C.Agardh taxa (25-30 μm), broad *Zygnema* C.Agardh taxa (22-40 μm), *Clastidium setigerum* Kirchner, *Teilingia granulata* (J.Roy & Bisset) Bourrelly, *Lemanea fluviatilis* (Linnaeus) C.Agardh and several *Spirogyra* Link taxa. Prominent taxa indicating circumneutral and nutrient-rich conditions (top right hand corner of Fig. 3a) are *Chamaesiphon polymorphus* Geitler, *Chamaesiphon incrustans* Grunow, *Audouinella hermannii* (Roth) Duby, *Stigeoclonium tenue* (C.Agardh) Kützing, broad *Oedogonium* Link ex Hirn taxa (>29 μ) and some *Spirogyra* Link taxa.

A total of 418 diatom taxa have both TDI and DAM indicator values and consequently are used in the UK for eutrophication and acidification assessment. The general picture is the same as

described above for the non-diatom benthic algae, i.e. taxa indicative of circumneutral conditions can be associated with nutrient-poor or –rich conditions whilst taxa associated with low pH generally also are associated with nutrient-poor conditions (Fig. 3b). Prominent taxa associated with acidic and nutrient-poor conditions include *Eunotia exigua* (Bréb.) Rabenhorst, *Peronia fibula* (Bréb ex Kütz.) Ross 1956 and *Pinnularia subcapitata* Greg. 1856. Important taxa associated with circumneutral and nutrient-rich conditions are *Amphora pediculus* (Kütz.) Grun. ex A. Schmidt and *Diatoma vulgare* Bory and prominent taxa associated with circumneutral and nutrient-poor conditions are *Hannaea arcus* (Ehrenb.) Patr. in Patr. & Reimer and *Achnanthes petersenii* Hust.

The current Swedish indices include 671 diatom taxa which have an index value both for the IPS and for the ACID calculation. The general picture is similar to that for Norwegian and UK indices, i.e. taxa indicative of circumneutral conditions can have either high or low IPS indicator values, whilst taxa indicating acid conditions usually also have a high IPS value, meaning they are indicative of undisturbed conditions and usually associated with low nutrient concentrations (Fig. 3c). There are, again, very few exceptions to this rule; for example *Nitzschia paleaeformis* Hust. and *Pinnularia sinistra* Krammer are associated with acidic conditions though they have moderate IPS sensitivity values, indicating tolerance to elevated nutrient and/or organic pollution levels. Prominent diatom taxa associated with acidic and nutrient-poor conditions almost all belong to the genus *Eunotia* Ehrenberg. At the other end of the scale taxa such as *Halamphora veneta* (Kützing) Levkov or *Achnantheidium exile* (Kützing) Heiberg occur in alkaline polluted waters usually with a high nutrient content. Examples of taxa which indicate circumneutral/alkaline conditions in ACID and rather nutrient-poor conditions in the IPS are *Rhopalodia gibba* (Ehrenberg) O. Müller and *Gomphonema olivaceum* (Hornemann) Brébisson.

3.2 Consistency among pH and nutrient indices

All three acid condition indices were significantly correlated to each other and to pH, both in the full dataset and the “acidity only” subset (Table 2). Correlation coefficients between the three indices were slightly higher in the “acidity only” subset than in the full dataset, whilst correlation coefficients between the indices and pH were slightly lower in the “acidity only” subset. This indicates that, if anything, consistency between acid conditions indices might be

slightly underestimated in datasets where TP and pH co-vary. AIP was the only acid conditions index which also was correlated to TP concentration, but only weakly so and only in the full dataset, not in the “acidity only” subset.

All four nutrient indices were significantly correlated to each other and to TP, both in the full dataset and the “nutrients only” subset (Table 3). With the exception of PIT-TI, correlation coefficients between nutrient indices were lower in the “nutrients only” subset than in the full dataset (Table 3) suggesting that the consistency between nutrient indices may be overestimated in datasets where TP and pH co-vary. All four nutrient indices were also correlated to pH in the full dataset, and the TI was even better correlated to pH than to TP. In contrast, there was no correlation between the nutrient indices and pH in the “nutrients only” subset (Table 3).

Diatom acid condition indices were better correlated with each other than to the non-diatom based AIP. This is not surprising, since all diatom indices are calculated from the same species list, so any variability amongst diatom indices stems exclusively from differences in species indicator values, whilst the variability between diatom and non-diatom indices additionally includes autecological differences between taxa. This, however, did not apply to the nutrient indices, especially not in the “nutrients only” subset, where the PIT correlated better to both TI and TDI than the diatom indices correlated to each other (Table 3). With few exceptions, the IPS generally had the lowest correlation coefficients to the other nutrient indices and to TP. This was expected, as the IPS was constructed to integrate the effects of nutrients with the effects of organic pollution (Coste in Cemagref, 1982), while TI, TDI and PIT were all designed specifically to indicate eutrophication via phosphorus concentrations.

3.3 Inter-relationships between pH and TP and respective indices

Maximum values of TP (i.e. the trendline reflecting the upper 95th percentile of the dataset) were associated with high pH, due to a lack of sites with both low pH and high nutrient concentrations (Fig. 4a). This is reflected in all benthic algae indices (Figs. 4b-d). A difference between chemistry and indices occurred, however, when nutrient concentrations were low. Minimum TP concentrations (i.e. the 5th percentile) were around 2 µg/l and decreased slightly as pH increased (Fig. 4a). In contrast, minimum values of PIT increased with increasing AIP (Fig. 4b), and minimum values of TDI increased with increasing DAM (Fig. 4c). However, maximum values of IPS (reflecting nutrient-poor conditions and no organic pollution)

decreased only slightly with increasing ACID (Fig. 4d). The response of minimum values of PIT and TDI, and maximum values of IPS against pH indices is the same in the “acidity only” dataset (data not shown). We show here only the plots for a comparison of within-country indices; however, all plots of pH and acid conditions indices against nutrient indices showed the same picture: minimum values of nutrient indices PIT, TDI and TI increased with increasing pH, AIP, ACID and DAM whilst maximum values of IPS decreased slightly. Overall, our results suggest that the lowest possible values of nutrient indices tend to be lower at acid, compared to circumneutral or alkaline sites.

3.4 Influence of TP and pH on diatom and non-diatom benthic algae richness patterns

For both non-diatom taxon richness, as well as diatom taxon and genus richness, only the linear term for $\log_{10}(\text{TP})$ was retained in the multivariate models (Table 4) whilst the polynomial term for pH explained more of the variation than the linear term (as indicated by the standardized regression coefficients; Table 4). To examine the influence of a skewed dataset on univariate richness patterns, we therefore fitted the response of richness to pH to a polynomial term, and to $\log_{10}(\text{TP})$ to a linear term, both for the full dataset, and the “nutrients only” and “acidity only” subsets (Fig. 5).

Non-diatom taxon richness was significantly related to a hump-shaped function of pH, a decreasing function of $\log_{10}(\text{TP})$ and an increasing function of their interaction (Table 4). $\log_{10}(\text{TP})$ had a stronger influence on non-diatom richness than pH, but the effect size of the interaction term was strongest (as indicated by the standardized regression coefficients; Table 4). 48% of the total variability in non-diatom taxon richness was explained by pH and $\log_{10}(\text{TP})$ (adjusted R^2 for the top model = 0.48). Average maximum non-diatom taxon richness was reached at around pH 6.4 (Fig. 5b) and at low TP concentrations (Fig. 5c). At higher TP concentrations, however, the observed pH maximum in non-diatom taxon richness had a lower value and its position was shifted towards more circumneutral conditions (Fig. 6a). The combined effect of increased nutrients and lower pH increased taxon richness less (at high pH) or decreased it more (at low pH) than would be expected if the effect was additive. This is consistent with a synergistic interaction between nutrient supply and low pH. In acidic rivers, nutrient enrichment had a stronger negative effect on ecosystem structure (measured as decrease in non-diatom benthic algae taxon number) than in circumneutral rivers (Fig. 6a). The general pattern for the univariate response of non-diatom taxon richness to both pH and $\log_{10}(\text{TP})$ was

the same in the full dataset and the “acidity only” subset, but R^2 was higher for pH in the “acidity only” subset compared with the full dataset, whilst it was lower for $\log_{10}(\text{TP})$. Note that the univariate analysis of the response of non-diatom taxon richness to pH did not result in a significant result in the full dataset (Fig. 5a). The significant response of non-diatom taxon richness to pH only became evident when the nutrient gradient was either removed (Fig. 5b) or accounted for in a multivariate model (Table 4). These results indicate that non-diatom richness patterns to pH may be overlooked when the effect of TP is not accounted for, whilst the richness in relation to $\log_{10}(\text{TP})$ is likely to be overestimated unless the effect of pH is also considered.

Diatom taxon and genus richness was best explained by a hump-shaped function of pH and an increasing function of $\log_{10}(\text{TP})$ (Table 4). However, only the hump-shaped relation of diatom genus richness to pH was significant in the multivariate model (as indicated by the confidence intervals of the standardized regression coefficients; Table 4). In contrast to non-diatom taxon richness, pH explained more of the variation than $\log_{10}(\text{TP})$ (as indicated by the standardized regression coefficients; Table 4). About 26% of the total variability in diatom taxon richness and 30% of diatom genus richness was explained by pH and $\log_{10}(\text{TP})$ (adjusted R^2 for the top models = 0.26 and 0.30, respectively, compared to 0.48 for non-diatoms), such that pH and $\log_{10}(\text{TP})$ explained less of the variation in diatom richness than of the variation in non-diatom richness. We detected no interaction between $\log_{10}(\text{TP})$ and pH for diatom richness (Table 4) suggesting that, in contrast to non-diatom benthic algae, diatom richness follows a simple additive pattern. Maximum diatom taxon and genus richness occurred at around pH 6.9 and 6.6, respectively, and at the upper end of the TP gradient in our data (TP 100 $\mu\text{g L}^{-1}$; Fig. 5). Since there was no interaction term between $\log_{10}(\text{TP})$ and pH on diatom richness, the maximum value of the relationship did not change with increasing TP concentrations (Figs. 6b, c). Consequently, the general pattern for the univariate response of non-diatom taxon richness to both pH and $\log_{10}(\text{TP})$ was similar in the full dataset and the subsets (Fig. 5).

4. Discussion

All indices of acid conditions were significantly correlated with each other and with pH, as were all nutrient indices with each other and with TP. This was true for both the full dataset and the subsets (Tables 2, 3). We thus conclude that pH and TP affect assemblages of diatoms and non-diatom benthic algae in a predictable and comparable way.

4.1 Confounding factors for indices and consequences for stream bioassessment

Our results confirm the hypothesis outlined in the introduction: that pH-gradients confound the interpretation of benthic algal richness patterns and indices designed to reflect nutrients. Most biotic indices encapsulate associations between organisms and chemistry derived from spatial surveys and, as a consequence, will be vulnerable to factors which co-vary with the pressure gradients on which the indices are calibrated. In the case of nutrients and acid conditions, there are too few instances where low pH and elevated nutrients are combined, meaning that the two stressors acidification and eutrophication are not, in reality, independent from each other. Even though areas on Ca-poor bedrock are used for some types of agriculture and forestry in northern Europe, intensive agriculture and urban settlement, the major contributors of phosphorus to freshwater ecosystems (Ulen et al., 2007), mainly takes place in comparatively base-rich areas, which are buffered, to a large extent, against the detrimental effects of acid deposition. Conversely the most severe acidification effects are seen in areas with siliceous bedrock types, and thin and podsollic soils (Henriksen, 1979), which are poorly suited for agriculture. Consequently, surface waters in these areas have a lower risk of being exposed to heavy eutrophication. This situation was also reflected in our water chemistry data: sites with low pH tended to also have low TP concentrations, whilst circumneutral sites can have both high and low TP concentrations.

There are two consequences of this coupling between nutrient concentrations and pH, and the relative rareness of nutrient rich-low pH sites in general: 1) indices developed from spatial surveys include few, if any, acidic indicator species which at the same time are indicators for high nutrient supply, and 2) taxon specific nutrient indicator values calculated from such surveys by averaging are likely to be lower for acid-tolerant species than for acid-sensitive species. This, in turn, will lead to the lowest possible inference of trophic status at a site being lower at acid than at circumneutral or alkaline sites (Figs. 4b-d; note: the scale is reversed for the IPS, so low pH sites will yield higher values). This is most likely an artifact of the datasets used for setting up the different indices rather than a true difference in ecological niche with respect to nutrients. For example, data from spatial surveys lead to *Zygonium* Kützing sp. and *Eunotia exigua* (Brébisson ex Kützing) Rabenhorst being associated with acidic, nutrient-poor conditions (van Dam et al. 1994; Rott et al., 1999; Kelly et al., 2008; Schneider & Lindstrøm, 2009; 2011). Hargreaves et al. (1975), however, found these taxa also at highly

acidic sites with PO₄-P-concentrations up to 0.9 (*Zygonium* sp.) and 1.8 mg/l (*Eunotia exigua*).

There are serious consequences for monitoring nutrient stresses in softwater environments where there may be a concomitant pH stress. Naïve use of an index such as the PIT, TDI, TI or IPS might indicate changing nutrient status when, in fact, it is the degree of acid stress which is fluctuating. A large body of literature exists, describing differences in diatom assemblages caused by natural differences in pH related to ecoregions (e.g. Tison et al., 2005; Lavoie et al., 2006). We here go one step further, saying that acidification alone can influence the outcome of nutrient indices, such that the uninformed use of indices in a multiple stressor situation might lead to acidification being interpreted as reduced nutrient concentrations or recovery from acidification as eutrophication. This is further complicated by the interplay between acid and nutrient stresses: many upland freshwater ecosystems are N-limited (Maberley et al., 2003) and increases in pH are mostly due to reductions in sulphate, rather than nitrate deposition (Flower et al., 2010), such that nitrogen concentrations in these ecosystems still are relatively high. Most nutrient indices, however, are calibrated on phosphorus concentrations, while the influence of nitrogen on these indices is largely unknown. There is, in short, a very real need in these situations to be able to monitor ecological responses to both acidification and nutrient stresses, at the same time as the limitations in existing indices are made clear. Similarly, acid deposition has not, until now, been explicitly included in screening protocols for reference sites (e.g. Pardo et al., 2012) whereas our evidence with respect to surface water pH suggests that it may affect “expected” index values not just for benthic algae but also for other taxonomic groups known to respond to both nutrient/organic and acid pressures (Bennett et al., 2011; Moe et al., 2010; Schartau et al., 2008). Note that Sweden, UK and Norway all excluded sites known to suffer anthropogenic acidification from the dataset reported in Kelly et al. (2012).

Although covariance might lead to indices being correlated with parameters other than the parameter of interest (e.g. nutrient indices being correlated with pH), this does not automatically mean that the indices do not provide useful insights into that parameter. Correlation coefficients between nutrient indices and TP, as well as acid conditions indices and pH were barely affected when the confounding factor was removed (Tables 2, 3). The most severe consequences of the confounding factor were a) the decreased consistency between the different nutrient indices rather than between these and TP, and b) the minimum recorded values of nutrient indices drifting with pH, possibly leading to acidification being interpreted as reduced nutrient concentrations. While the consistency among nutrient indices in datasets including both a

nutrient and a pH gradient is overestimated, this was not the case for the consistency among indices of acid conditions. This may be because pH exerts a greater physiological stress than nutrients, such that both diatom richness and species composition (Andrén & Jarlman, 2008; Fisher et al., 2010) are more closely connected to pH than to nutrient supply.

4.2 Effects of pH and nutrients on taxon richness

In order to better understand the underlying structural responses of benthic primary producers to acid conditions and nutrient supply, we also analyzed benthic algae richness patterns. Diatom and non-diatom samples at each study site were taken at the same time, so diatoms and non-diatoms were exposed to identical environmental conditions. Consequently, differences in diatom and non-diatom richness patterns must be explained by ecological differences among these two groups rather than by external environmental conditions. The diatom assemblage is often treated as a proxy for the whole stream phyto-benthos, while non-diatom benthic algae received considerable less attention (Kelly, 2006; Kelly et al., 2009). Our data show, however, that stream diatom and non-diatom benthic algae taxon richness show very different patterns in relation to nutrient supply and pH.

Effects of pH

Richness of both diatom and non-diatom benthic algae has a hump-shaped pattern in relation to pH, but non-diatom benthic algae reached their highest taxon richness around pH 6.4, while diatom richness had its maximum around pH 6.9 (Fig. 5b). Different factors associated with low pH (elevated H^+ , elevated labile Al concentration, low concentration or absence of HCO_3^- , reduced solubility of quartz (Stumm & Morgan, 1996), different benthic macroinvertebrate taxon richness and feeding types (Larranaga et al, 2010)) all impact benthic algae, and discriminating between the effects of these factors on primary producers is difficult (Sparling & Lowe, 1996). Eukaryotic algae have developed a variety of mechanisms for inorganic carbon acquisition, including utilization of HCO_3^- and different carbon concentrating mechanisms (Raven, 2010). The observed maximum in non-diatom taxon richness around pH 6.4 is very close to the point where equilibrium concentrations of CO_2 and HCO_3^- in open water systems are equal (Stumm & Morgan, 1996), suggesting the coexistence of bicarbonate and CO_2 users.

Note, however, that the observed maximum in non-diatom taxon richness towards pH shifts with increasing TP concentrations (Fig. 6a). This shift is the reason why non-diatom taxon richness patterns towards pH might be overlooked in datasets which are skewed with respect to pH and TP (Fig. 5a, where the non-diatom richness pattern was not significant, while a significant pattern was detected when the nutrient gradient was removed (Fig. 5b) or accounted for (Table 4)).

In contrast to non-diatoms, diatom taxon richness increased between pH 5 and 6.9, which might be explained by bicarbonate utilization being widespread among diatoms (Martin & Tortell, 2008). Species-poor diatom assemblages in acid compared to circumneutral streams are a well-known phenomenon (Ledger & Hildrew, 2001); however, taxon richness-pH relationships for lake diatoms differ markedly among regions (Telford et al., 2006). Lakes in South Norway exhibit maximum diatom taxon richness at around pH 6.4, probably because most lakes in South Norway have a pH around this point (Telford et al., 2006). There are three possible explanations why the maximum diatom richness observed in our data is at a higher pH than observed by Telford et al. (2006): a) our data are from rivers, while Telford et al. (2006) analyzed data from lakes where C-limitation is likely to be more pronounced in the absence of flow-driven enhancement of oxygen efflux from the organism to the water, increasing the affinity of the RuBisCO enzyme to CO₂ (Mass et al., 2010); b) Telford et al. (2006) analysed only data from South Norway, whilst our data also include North Norway where more Ca-rich bedrock occurs (Skjelkvåle & Wright, 1990), leading to higher pHs than reported in Telford et al. (2006); and c) both higher TP and higher pH lead to an increase in diatom taxon richness (Fig. 5); if the data of Telford et al. (2006) included a nutrient gradient, their observed richness maximum might have been biased. All three different explanations actually might play a role.

Effects of nutrients

Nutrient enrichment in acidic rivers had a more negative effect on non-diatom benthic algae taxon number than in circumneutral rivers (Fig. 6a), perhaps indicating that acidic ecosystems are particularly sensitive to eutrophication.

Non-diatom taxon richness decreased with increasing TP, whilst diatom taxon richness increased. This contradicts expectations, since in freshwater ecosystems, fertilization is assumed to generally increase primary producer taxon richness (Hillebrand et al., 2007). Our

data indicate that non-diatom richness is more closely related to TP than to pH, while it is the other way round for diatom taxon richness (Table 4; see also Andrén & Jarlman, 2008; Fisher et al., 2010).

Increased nutrient supply decreased non-diatom benthic algae richness, and we suggest that this pattern can be explained by the classical concept of niche theory, where taxon richness decreases with increasing nutrient supply due to the exclusion of taxa by superior competitors (Stevens et al., 2004; Wassen et al., 2005). It has been suggested that larger algal species in periphyton communities, which often are favored by fertilization, provide additional habitat structure for epiphytic diatoms, thus leading to enhanced species richness in fertilized freshwater ecosystems (Hillebrand, 2003). This, however, seems to not work for epiphytic non-diatom algae, since non-diatom benthic algae decreased rather than increased in eutrophic sites. The different patterns we observed between diatom and non-diatom taxon richness in relation to nutrient supply might instead be explained by different tolerances to low light conditions, different motility and ability to utilize facultative heterotrophism. Adnate diatoms are inherently well adapted to low light conditions (Steinman et al., 1992), while chlorophytes typically require higher light intensities (Hill, 1996). In addition, many diatom species are motile and can regulate their light environment through phototaxis (Hill, 1996). Likewise, facultative heterotrophy is common among diatoms, which enables them to survive low light periods in the understory of larger algal taxa (Tuchman, 1996). All these mechanisms enable diatoms to grow in the understory of larger algal taxa, which are often favored by fertilization, while less well-adapted epiphytes among the non-diatom algae might be outcompeted.

Thus, in case of non-diatom benthic algae, our data contradict the assumption that fertilization consistently increases taxon richness of primary producers in freshwater habitats (Hillebrand et al. 2007). Earlier observations of an increasing richness of benthic primary producers in streams may be explained by diatoms often being either the only group of benthic algae studied in streams, or being analyzed to a greater taxonomic resolution than other groups. Diatoms are - except in very acidic sites - usually more species rich than non-diatom benthic algae (see Fig. 5), such that they are likely to override the signal of non-diatom benthic algae. Pooling diatoms and non-diatom benthic algae into one group will thus likely result in the observation of an either absent or slightly increasing pattern in total taxon richness with nutrient supply, as observed in the meta-analysis of Hillebrand et al. (2007).

A number of different factors interact in affecting benthic primary producer taxon richness, the most commonly investigated being nutrient supply and disturbance (Biggs & Smith, 2002; Cardinale et al., 2006), as well as dispersal limitations (Matthiessen et al., 2010). We show here that pH is an important additional factor influencing primary producer richness, and that underlying gradients in pH, when not accounted for, interfere with patterns in benthic primary producer richness to nutrient supply. A covariance between nutrient- and pH-gradients introduces considerable variability into analysis of biological response to one factor unless the other is accounted for.

Consequences of the different richness patterns

What are the consequences of the different richness patterns between diatoms and non-diatoms? Diatoms and non-diatom benthic algae compete, after all, for the same nutrients, with the likely exception of silica (Carrick & Lowe, 2007). Nevertheless do the observed differences in taxon richness patterns suggest that mechanistic explanations might be different for diatoms and non-diatom benthic algae. The search for an explanation of observed patterns in diversity-productivity relationships in meta-analysis (Whittaker, 2010; Cardinale et al., 2011) might therefore be complicated by the fact that some studies deal exclusively with diatoms, whilst others pool diatoms and non-diatom benthic algae richness. Our results are consistent with the results of Schneider et al. (2012), who described differences in stream diatom and non-diatom benthic algae community patterns, and suggest that benthic primary producers in streams are not the homogenous group they often are treated as. Moreover, whilst diatom and non-diatom indices both are suitable for status assessment, their assemblage patterns follow different ecological principles.

Good ecological status according to the Water Framework Directive is defined via ecosystem structure and functioning, i.e. good ecological status is supposed to reflect a resilient ecosystem with a high level of adaptive capacity (Josefsson & Baaner, 2011). Ecosystem productivity and resource use efficiency are important ecosystem functions, and the general view today is that both generally depend on primary producer taxon richness, though variations in the shape of the productivity-diversity relationship are observed among individual studies (Mittelbach et al., 2001; Ptasnik et al., 2008). Our data suggest that the relative influence of diatoms and non-diatom benthic algae on ecosystem structure and functioning will vary in response to both pH

and nutrient supply. Consequently, accurate assessment of phyto-benthos structure and function might require analysis of both diatoms and non-diatom benthic algae. Irrespective of the observed differences between diatoms and non-diatom benthic algae, however, each assemblage is generally linked to nutrient supply as well as pH, and the resulting indices do provide a direct link to these pressures, making them valuable tools for river basin management. At the same time we here show that pH gradients add another element of complexity to the challenge of understanding ecosystem responses to multiple stressor situations (Ormerod et al., 2010).

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Figure captions

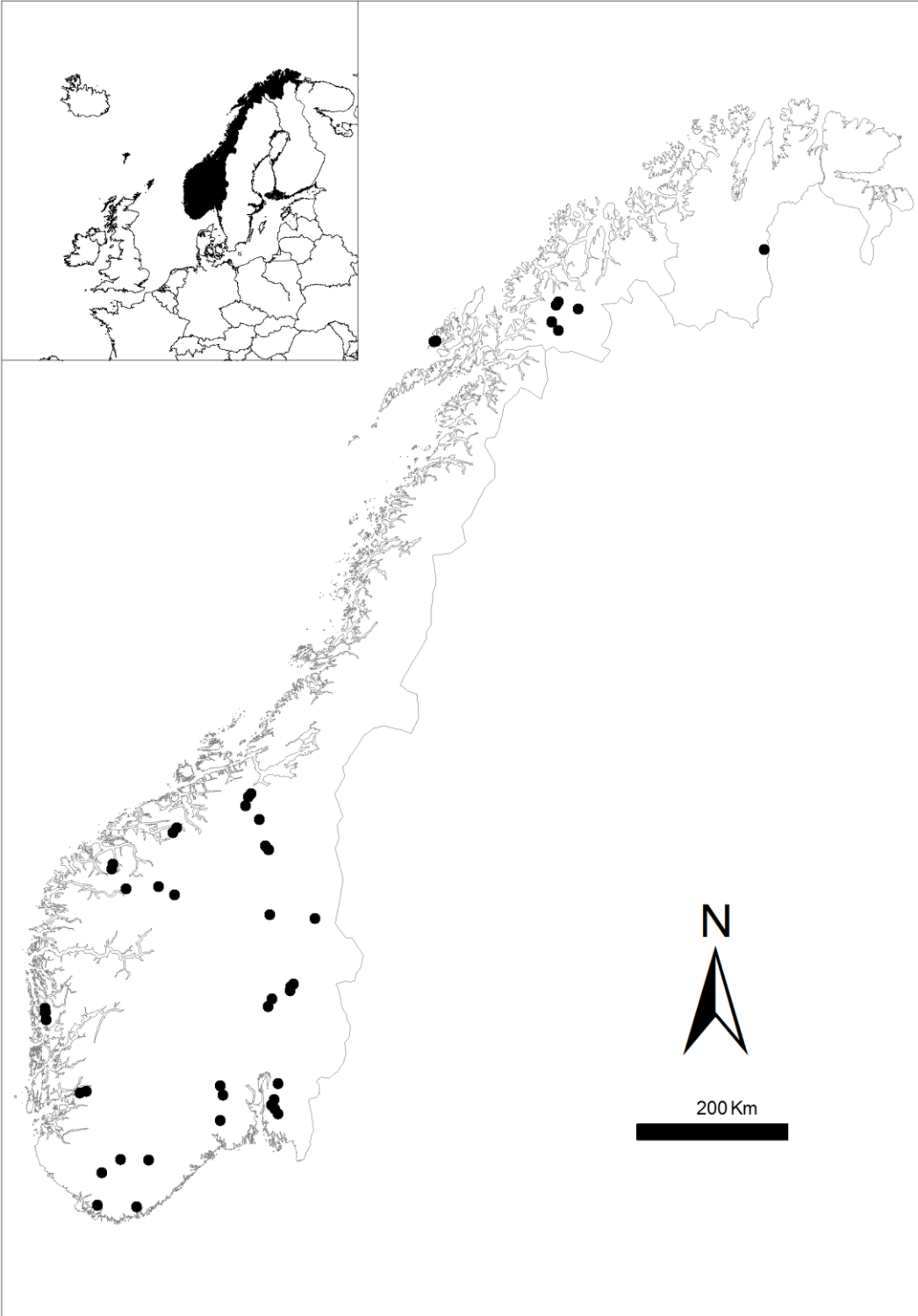


Fig. 1. 52 river sites were sampled in Norway between 1981 and 2007.

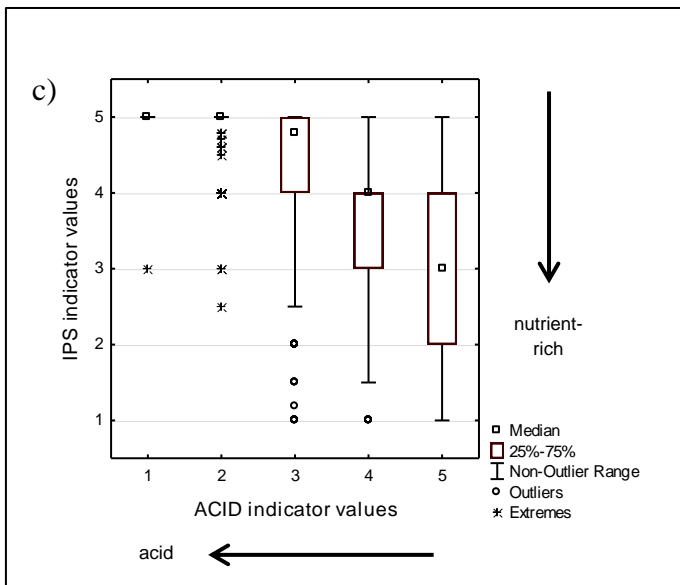
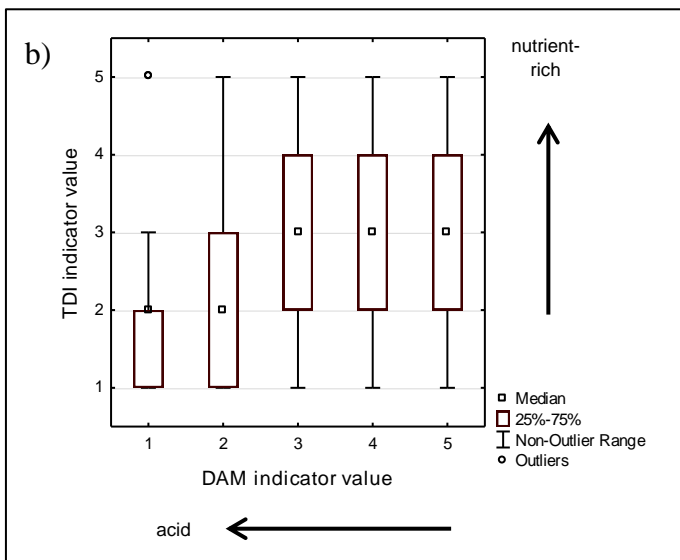
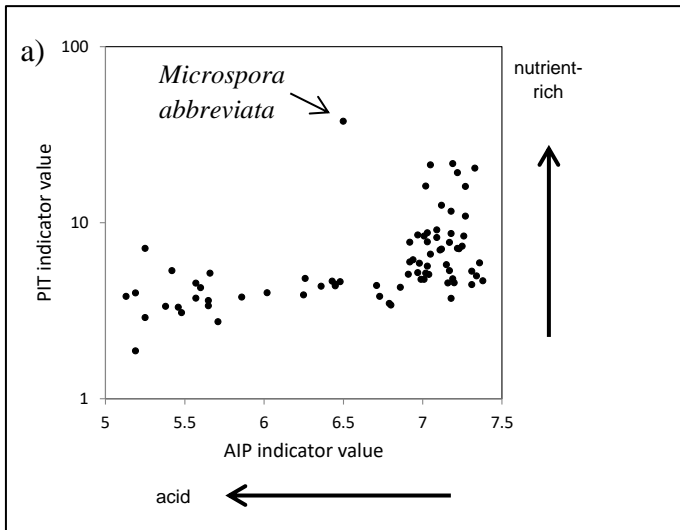


Fig. 3. Taxa specific index values for pH plotted against nutrients (general pollution in case of IPS); (a) 77 non-diatom benthic algae taxa which are indicative in both AIP and PIT

(Norwegian assessment system); (b) 418 diatom taxa which are indicative in both DAM and TDI (UK assessment system); (c) 671 diatom taxa which are indicative in both ACID and IPS (Swedish assessment system). Low ACID, DAM and AIP values generally denote acid-tolerant taxa, while high PIT and TDI values denote taxa preferring high nutrient concentrations; however, high IPS values denote pollution sensitive taxa, i.e. taxa usually associated with nutrient poor conditions.

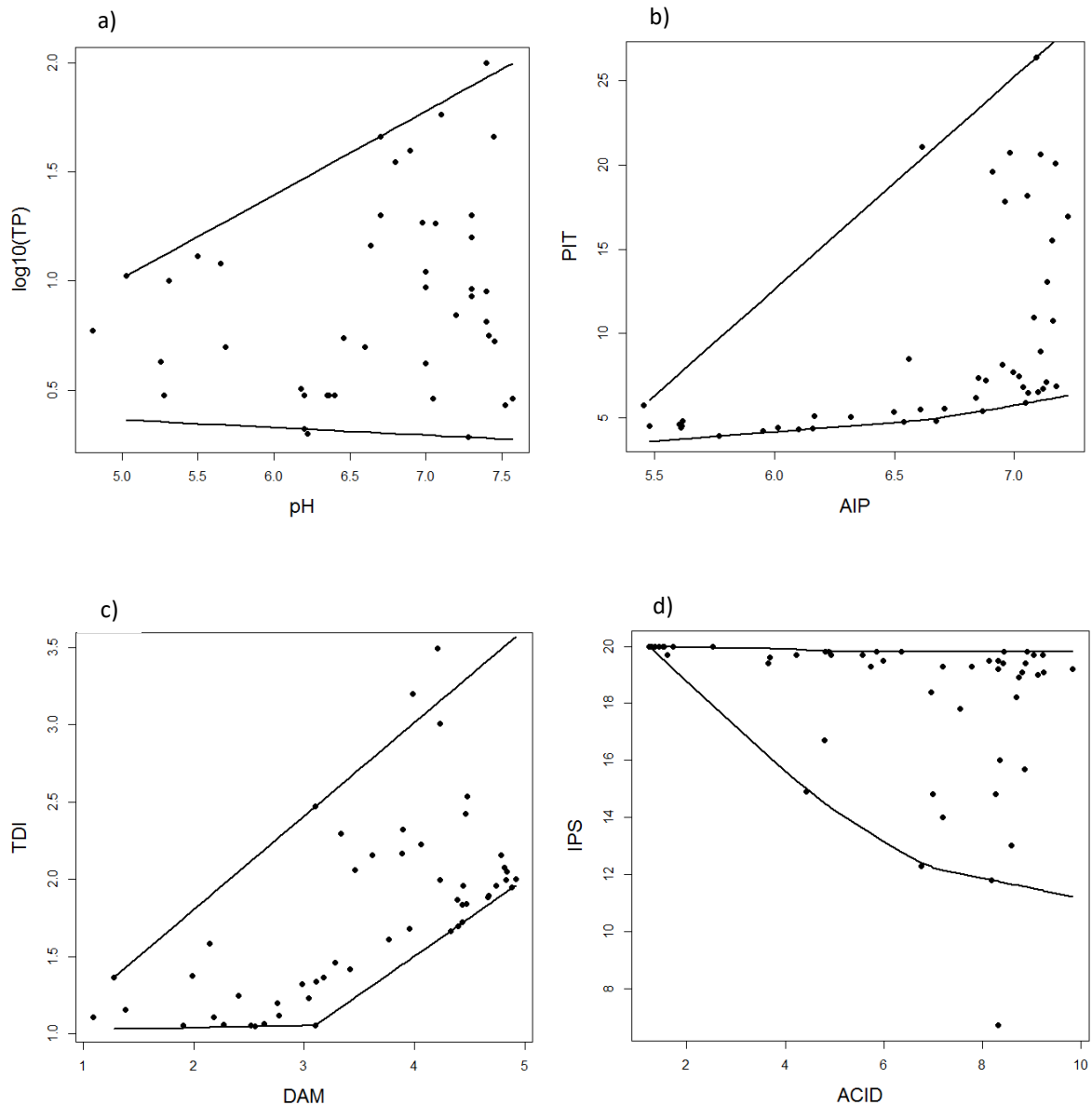


Fig. 4. Acid conditions plotted against nutrient supply at 52 sites in Norway; (a) pH against total phosphorus concentration (TP, in $\mu\text{g/l}$); (b) Norwegian bioindication methods: AIP (acidification index periphyton) against PIT (periphyton index of trophic status); (c) UK bioindication methods: DAM (diatom acidification metric) against TDI (trophic diatom index); (d) Swedish bioindication methods: ACID (Swedish diatom pH index) against IPS (Indice de Polluo-sensibilité Spécifique); Lines indicate 95th and 5th percentile.

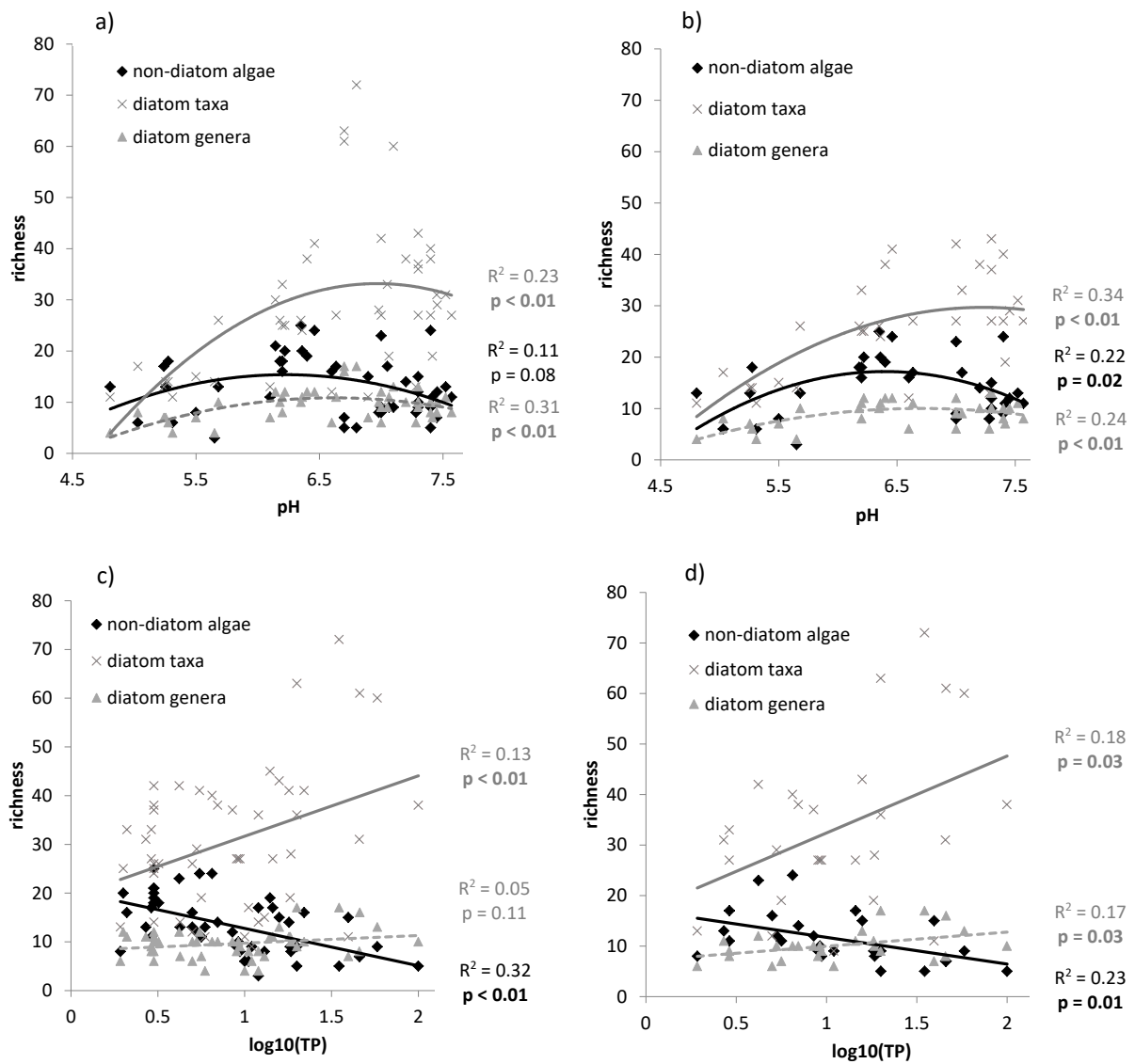


Fig. 5. Non-diatom algae taxon richness, diatom taxon and genus richness plotted against pH and log₁₀(TP) (in µg/l). Left hand figures (a, c): full dataset; (b) “acidity only” subset, (d) “nutrients only” subset. Lines indicate second order polynomial models for pH, and linear models for logTP; significant p-values (ANOVA) are in marked in bold.

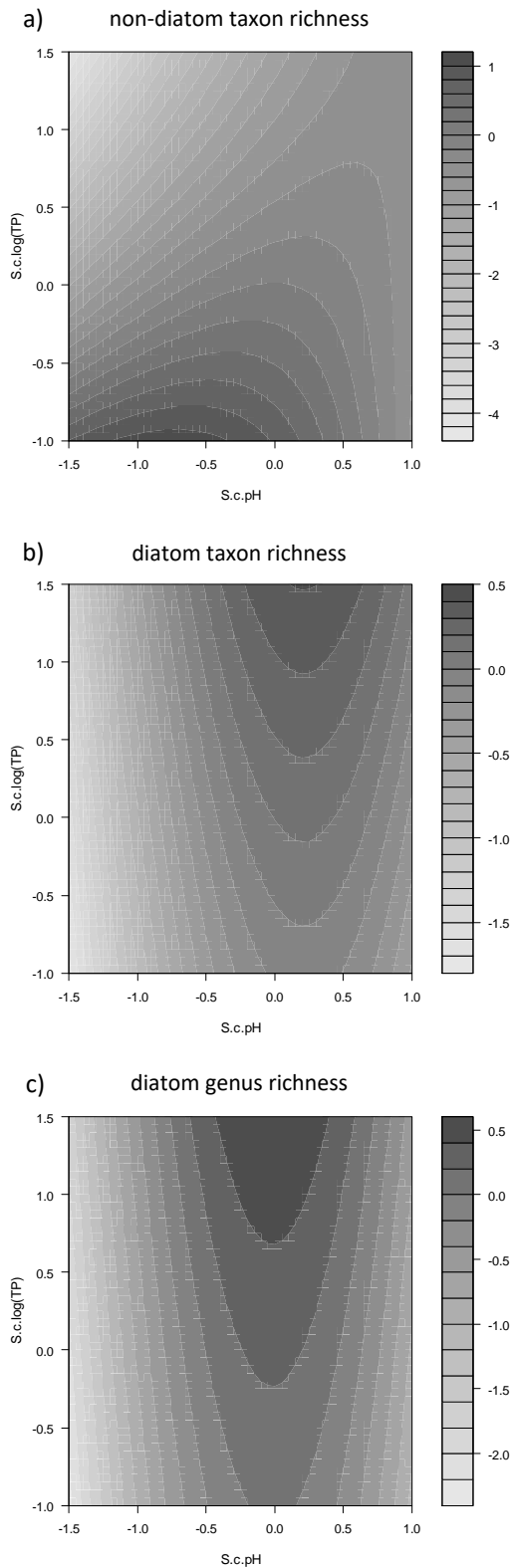


Fig. 6. Relationships between TP, pH and taxon richness. Note that all data are centered and scaled, that means a value of zero represents the sample average, while positive and negative values are larger and smaller than the average, respectively. pH: mean = 6.60, SD = 0.77; logTP: mean = 0.92, SD = 0.43. S.c.pH = centered and scaled pH, S.c.log.TP = centered and scaled logTP. Dark colors indicate higher taxon numbers.

	minimum	average	maximum
cond ($\mu\text{S}/\text{cm}$)	9.0	61.8	290.0
Ca (mg/l)	0.3	5.2	24.4
TOC (mg/l)	0.5	4.2	17.1
Tot-N ($\mu\text{g}/\text{l}$)	46	508	4400
pH	4.8	6.6	7.6
Tot-P ($\mu\text{g}/\text{l}$)	1.9	13.9	100.0
AIP	5.5	6.6	7.2
PIT	3.9	9.2	26.5
ACID	1.2	6.1	9.8
IPS	6.7	18.3	20.0
DAM	1.1	3.6	4.9
TDI	1.0	1.8	3.5
TI	0.6	1.3	3.0
taxon number non-diatom algae	3.0	13.6	25.0
taxon number diatoms	9.0	30.0	72.0
genus number diatoms	4.0	9.6	17.0

Table 1. Summary statistics of abiotic variables and biotic indices at 52 sites in Norway. AIP = acidification index periphyton; DAM = diatom acidification metric; ACID = Swedish diatom pH index; PIT = periphyton index of trophic status; IPS = Indice de Polluo-sensibilité Spécifique; TDI = trophic diatom index; TI = Trophieindex.

	AIP	ACID	DAM	pH
ACID	0.80 (0.86)			
DAM	0.82 (0.84)	0.95 (0.96)		
pH	0.91 (0.90)	0.66 (0.65)	0.66 (0.63)	
TP	0.39 (0.31)	0.23 (0.23)	0.18 (0.21)	0.16 (-0.05)

Table 2. Spearman rank correlation coefficients between acid condition indices, pH and total phosphorus (TP) for all 52 sites, and for the “acidity only” subset (in brackets); AIP = acidification index periphyton; DAM = diatom acidification metric; ACID = Swedish diatom pH index; bold values are significant at $p < 0.05$.

	PIT	IPS	TDI	TI	TP
IPS	-0.75 (-0.66)				
TDI	0.82 (0.79)	-0.80 (-0.54)			
TI	0.85 (0.88)	-0.90 (-0.77)	0.87 (0.69)		
TP	0.72 (0.75)	-0.63 (-0.61)	0.68 (0.69)	0.63 (0.67)	
pH	0.64 (-0.19)	-0.62 (0.15)	0.59 (-0.21)	0.74 (-0.01)	0.16 (-0.30)

Table 3. Spearman rank correlation coefficients between nutrient indices, TP and pH for all 52 sites, and for the “nutrients only” subset (in brackets); PIT = periphyton index of trophic status; IPS = Indice de Polluo-sensibilité Spécifique; TDI = trophic diatom index; TI = Trophieindex; bold values are significant at $p < 0.05$.

	non-diatom taxon richness			diatom taxon richness			diatom genus richness		
	standardized regression coefficient	CI (2.5%)	CI (97.5%)	standardized regression coefficient	CI (2.5%)	CI (97.5%)	standardized regression coefficient	CI (2.5%)	CI (97.5%)
intercept	0.01	-0.17	0.19	0.10	-0.10	0.30	0.25	0.04	0.45
pH	-0.01	-0.32	0.29	0.27	-0.08	0.62	-0.01	-0.37	0.35
pH ²	-0.55	-1.05	-0.06	-0.56	-1.15	0.03	-1.03	-1.64	-0.43
logTP	-0.76	-1.03	-0.50	0.19	-0.09	0.46	0.22	-0.06	0.50
pH*logTP	0.84	0.17	1.51						

Table 4. Standardized regression coefficients and their confidence intervals (CI) for the top model (non-diatoms) and averaged top 2 AIC_c models (diatoms). Note that standardized (centered and scaled) regression coefficients can be used as measures of effect size and that an inclusion of zero in the 95% CI of the regression coefficients means that the effect is not statistically significant at $\alpha = 0.05$. Significant coefficients are marked in bold.