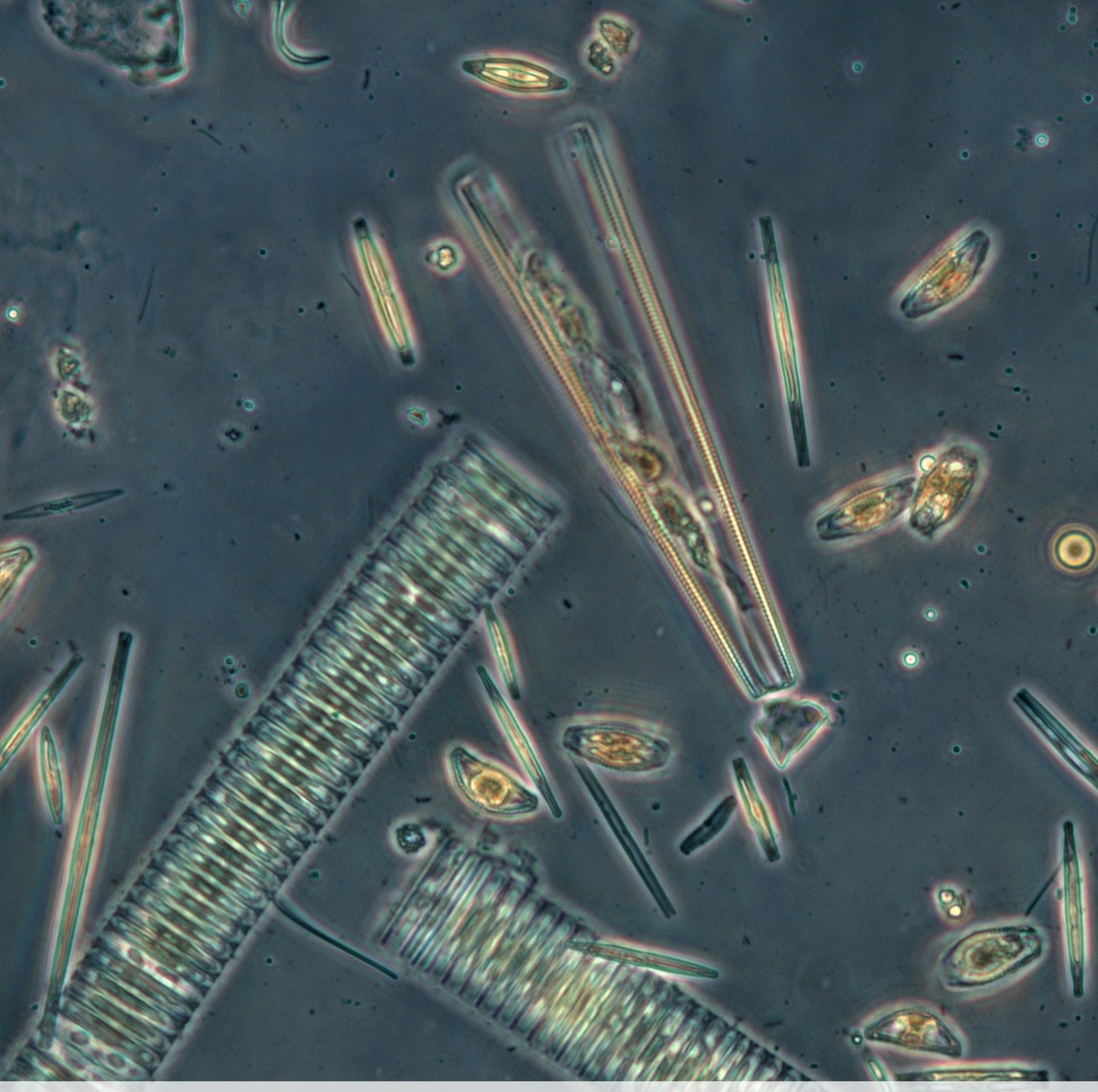

ALKALINITY AND ITS INFLUENCE ON BENTHIC DIATOM ASSESSMENTS IN DANISH RUNNING WATERS

Scientific Report from DCE – Danish Centre for Environment and Energy No. 521 2023

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Abstract:	Alkalinity and PO_4 co-varies in Danish running waters, and this affects the applicability of the diatom-based indicator of eutrophication SID_TID. Through the analysis of physicochemical and diatom databases, we evaluated the influence of alkalinity on the ability to meet ecological targets and sought to disentangle the effect of alkalinity by the abundance of target diatom species as indicator of high alkalinity and low PO_4. Alkalinity was correlated to trophic status indicators (PO_4 and BI5), confirming that it is an important interfering factor influencing the SID_TID, and we thus discuss potential causes of the association between these factors. We found no relationship of alkalinity with the abundance of target diatom species. We then developed a probability model, which indicated that at alkalinity values greater than 2,6 mEq/L the probability of meeting ecological targets based on the SID_TID declines drastically and, therefore, the convenience of this index at higher alkalinities should be evaluated.

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Preface

The current scientific report has been produced on request from the Danish Environmental Protection Agency (MST) with the aim to disentangle the role of alkalinity in affecting the ability to achieve good ecological status as assessed by the benthic algal index SID_TID for Danish watercourses. It continues the research into the effects of alkalinity as an interfering factor for achieving the environmental targets set for Danish watercourses using SID_TID, and includes two previous projects conducted for MST:

Andersen, D.K., Larsen, S.E., Johansson, L.S., Alnøe, A.B., and Baattrup-Pedersen, A., 2018. Udvikling af biologisk indeks for bentiske alger (fytobenthos) i danske vandløb. Aarhus Universitet, DCE – Nationalt Center for Miljø og Energi, 42 s. – Videnskabelig rapport nr. 296. http://dce2.au.dk/pub/SR296.pdf

and

Baattrup-Pedersen, A., Johnsen, T.J. and Riis, T. 2021. Betydningen af danske vandløbs alkalinitet for de bentiske algesamfund. Aarhus Universitet, DCE – Nationalt Center for Miljø og Energi, 34 s. - Videnskabelig rapport nr. 440 http://dce2.au.dk/pub/SR440.pdf

as well as the resulting manuscript:

Baattrup-Pedersen, A., Johnsen, T.J., Larsen, S.E. and Riis, T., 2022. Alkalinity and diatom assemblages in lowland streams: How to separate alkalinity from inorganic phosphorus in ecological assessments?. Science of the Total Environment, 823, p.153829.

The current project focuses on the effect of alkalinity levels on the applicability of the diatom-based SID_TID index and the potential use of target benthic diatom species, previously identified as indicators of high alkalinity and low orthophosphate (PO_4) levels, to elucidate whether PO_4 or alkalinity is the main reason for failure to meet environmental targets. To this end, we analysed a combined dataset from 2013-2020 of physicochemical and benthic diatom data from, respectively, the NOVANA and OMNIDIA databases.

The results of this project have been presented to the MST, who have had the opportunity to comment on a draft version of this report.

Sammenfatning

Alkalinitet samvarierer med PO_4 i danske vandløb, hvilket har betydning for anvendeligheden af det bentiske algeindeks SID_TID som indikator for menneskeskabt eutrofiering. I dette projekt er der anvendt data fra NOVANA- og Omnidia-databaser for perioden 2013 til 2020 fra danske vandløb med henblik på i) at analysere, i hvor høj grad alkalinitet påvirker muligheden for at opfylde økologiske mål for vandløb vurderet med SID_TID, og ii) at undersøge muligheden for at adskille betydningen af alkalinitet og PO_4 for tilstandsvurderingen baseret på forekomsten af specifikke bentiske kiselalgearter, der tidligere er fundet hyppigt forekommende ved lav PO_4, og hvis forekomst samtidig øges med stigende alkalinitet. Nærværende resultater bekræfter tidligere resultater, der viser høj samvariation af PO_4 og alkalinitet, og at korrelationen mellem alkalinitet og SID_TID var højere end for alkalinitet og PO_4, hvilket tyder på, at alkalinitet influerer på tilstandsvurderinger med anvendelse af SID_TID. Der kunne dog ikke etableres sammenhænge mellem specifikke bentiske kiselalgearter og alkalinitet, og derfor kunne disse arter ikke bruges til at adskille virkningen af alkalinitet fra virkningen af PO_4 for tilstandsvurderingen i danske vandløb. I stedet blev der anvendt en alternativ tilgang til at identificere vandløb, hvor alkalinitetsniveauet kan påvirke den økologiske tilstand vurderet med SID_TID-indekset, og dermed influere på muligheden for målopfyldelse. Ved at anvende modelestimater fra en lineær regressionsanalyse blev der genereret en model, der kan anvendes til at forudsige sandsynligheden for at opnå god økologisk tilstand ved anvendelse af SID_TID langs en alkalinitetsgradient. Det opnåede resultat indikerede et drastisk fald i sandsynligheden for opfyldelse af god økologisk tilstand baseret på SID_TID (< 2,39) i vandløb med alkaliniteter højere end 2,6 mEq/L. På baggrund af disse resultater opfordres derfor til, at der tages højde for alkalinitetsniveauet i tilstandsvurdering af danske vandløb.

Summary

Alkalinity co-varies with PO_4 in Danish running waters, and this affects the applicability of the diatom-based ecological indicator SID_TID to assess anthropogenic eutrophication. Here, NOVANA and Omnidia databases on Danish watercourses from 2013 to 2020 were combined to i) analyse to what extent alkalinity influences the ability to meet ecological targets in running waters using SID_TID and to ii) explore the possibility of disentangling the effect of alkalinity from that of PO_4 based on the abundance of specific benthic diatom species previously identified to be abundant at low PO_4 and to increase in abundance with increasing alkalinity. The results confirm previous findings of the high co-variation of PO_4 and alkalinity, and that the correlation between alkalinity and SID_TID was even higher than for alkalinity and PO_4, indicating that alkalinity is an important interfering factor influencing SID_TID assessments. However, relationships between specific benthic diatom species and alkalinity could not be established, and therefore these species could not be used to disentangle the effect of alkalinity from that of PO_4 in Danish running waters. Instead, another approach was chosen to identify watercourses where alkalinity might influence the chance of meeting ecological targets using SID_TID. By applying model estimates derived from a linear regression analysis from empirical data, we generated a probability-based threshold for reaching good ecological status applying SID_TID along a gradient in alkalinity. This indicated a drastic decline in the probability of compliance with good ecological status based on SID_TID (< 2.39) in watercourses with alkalinities higher than 2.6 mEq/L. Based on these results, we advocate for taking into consideration the level of alkalinity in Danish running waters in management planning.

1 Introduction

1.1 Background

Diatoms are an abundant and diverse taxonomic group within the community of benthic autotrophs in running waters. Due to their high diversity and differential sensitivity to environmental conditions, diatoms have been widely applied to assess the ecological status of watercourses (Stevenson *et al.*, 2010; Dong *et al.*, 2015) in compliance with the Water Framework Directive (Schaumburg *et al.*, 2004; Kelly *et al.*, 2008; Poikane *et al.*, 2016). The existing diatom-based water quality indices have mainly focused on evidencing organic contamination and human-driven eutrophication with particular focus on orthophosphate (PO_4) as the main pollutant and promoter of primary production in aquatic ecosystems (Steinberg and Schiefele, 1988; Kelly and Whitton, 1995; Rott *et al.*, 1997, 1999, 2003).

These indices used are based on the responses of diatom species along a contaminant gradient, e.g., a PO_4 gradient, but there are other associated factors that may affect the direct interpretation of their results. Chemical changes during eutrophication or salinisation processes in freshwaters may induce the co-variation of human-driven nutrient increases along with changes in pH or alkalinity, which are influenced by natural conditions such as soil type and geology (Wiegleb *et al.*, 2005; Demars and Edwards, 2009). Alkalinity in aquatic ecosystems is the buffering capacity linked to the proportion of inorganic and organic acids and bases in water, which at neutral to alkaline pH are mainly bicarbonate and carbonates. Under natural conditions, alkalinity is determined by soil characteristics in the catchment that releases these ions to the water. In Denmark, alkalinity is typically low in sandy, lime-poor soils in West Jutland, while it is high in the more clayey and calcareous soils of Eastern Jutland and on the islands (Rebsdorf *et al.*, 1991). However, alkalinity can be influenced by human activities such as liming, fertilisation, urbanisation, and mining (Raymond and Cole, 2003; Kaushal *et al.*, 2013). Depending on its origin, alkalinity may affect the carbon source for diatoms due to changes in the relative availability of the different forms of inorganic carbon as well as the proportion of cations to anions (e.g., NH_4^+, Na^+, K^+, Ca^{2+}, Mg^{2+}, Cl^-, SO_4^{2-}, NO^{3-}) (Kaushal *et al.*, 2013). When alkalinity increases, bicarbonate (HCO_3^-) becomes increasingly important as a carbon source (Stumm and Morgan, 1995). Diatom species possess different metabolic mechanisms to concentrate and use these diverse carbon sources (Young *et al.*, 2016; Clement *et al.*, 2017). Therefore, the diatom composition will change along an alkalinity gradient depending on the efficiency of the metabolic system of each specie to the predominant carbon source (Clement *et al.*, 2017). Both alkalinity and eutrophication can co-vary and thus influence the diatom composition (Stumm and Morgan, 1995; Clement *et al.*, 2017), and proper application of these ecological indices relies on elucidating the differential effects of these associated co-variable factors.

The SID_TID index used in Denmark for the evaluation of the ecological status of watercourses is the average of two Austrian indices: the Saprobe index (SID; Rott *et al.*, 1997, 2003) and the trophic index (TID; Rott *et al.*, 1999) (SID_TID= SID+TID/2) (Andersen *et al.*, 2018). This index is correlated with the availability of PO_4 in water and was tested and intercalibrated in 2020 for catchments including a wide range of size classes (Andersen *et al.*, 2018). The limit between good ecological status and moderate status (SID_TID= 2.39) is based on the change point for diatom species that responds positively to increased PO_4 (Andersen *et al.*, 2018). Previous studies in Danish watercourses and in-stream experiments have shown that SID_TID is influenced by both PO_4 and alkalinity, since both can co-variate, which makes it difficult to disentangle the effects of human-driven eutrophication from those of alkalinity (Andersen *et al.*, 2018; Baattrup-Pedersen *et al.*, 2021; Baattrup-Pedersen *et al.*, 2022). In consequence, alkalinity affects the sensitivity of SID_TID as an indicator of PO_4. It has been found that even watercourses with low levels of PO_4 (0.004 mg/L) may exhibit high SID_TID values (SID_TID > 2.39) due to high alkalinities (Baattrup-Pedersen *et al.*, 2022). Therefore, it is key to separate the influence of alkalinity from PO_4 applying SID_TID to elucidate whether the main reason for failure to meet environmental targets assessed with this benthic diatom index is caused by PO_4 or by alkalinity.

1.2 Purpose

Previous studies have pointed out the relevance of water alkalinity as an interfering factor for achieving the environmental targets in Danish watercourses assessed through the benthic algal index SID_TID (Baattrup-Pedersen *et al.*, 2021; 2022). These studies also indicated that some specific diatom species are associated with high levels of alkalinity regardless of PO_4 levels. The abundance of these species may therefore allow us to separate the effect of alkalinity from that of PO_4 on SID_TID values. We therefore analysed to what extent the alkalinity of the stream water influences the probability of achieving good ecological status assessed with the benthic algal index, SID_TID based on all available data in NOVANA and OMNIDIA (2013-2020), and the ability of the above-mentioned diatom species to disentangle the influence of alkalinity from that of PO_4 on SID_TID.

2 Methods

2.1 Technical implementation

To analyse how alkalinity affects the applicability of the diatom-based SID_TID index used to assess ecological status in Danish running waters in response to eutrophication and to evaluate the potential use of target diatom species to isolate the effect of alkalinity from that of PO_4 on this index, the following steps were conducted:

- Compiling physicochemical and diatom data from Danish watercourses from Overfladevandsdatabasen (ODA) and data provided by the Danish Environmental Protection Agency/Miljøstyrelsen from the OMNIDIA database (Lecointe *et al.,* 1993) for the period 2013-2020 (https://omnidia.fr/en/).
- Analysing SID_TID index values in Danish watercourses, and how the SID_TID index varies along gradients of PO_4 and alkalinity.
- Analysing how benthic diatom species previously identified as being particularly abundant at high alkalinity (see table 1) respond to PO_4 to examine their potential use for disentangling the influence of high alkalinity from PO_4 conditions. Here, we analysed the abundance of these species individually as well as the overall abundance of these species along an alkalinity gradient to test correlations and critical levels of alkalinity, where these frequencies might change drastically.
- Developing an empirical relationship between SID_TID and alkalinity and applied model estimates to predict the probability of achieving minimum good ecological status (SID_TID < 2.39) to form the basis for an assessment of whether the naturally occurring alkalinity level can interfere with the ability to apply the SID_TID index to assess eutrophication.
- Reviewing the existing literature on the co-variation of alkalinity and inorganic orthophosphate in freshwaters and supplemented this with knowledge about the occurrence and frequency of the specific algae species along a gradient of alkalinity in watercourses.

Table 1. Diatom species previously identified as indicators of high alkalinity in Danish watercourses without influence from inorganic phosphorus. The indicator value for each species and the occurrence as a function of water alkalinity (expressed as a slope) * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. From Baattrup-Pedersen *et al.* (2021).

Species abbreviation	Species name	Indicator value for alkalinity	Regression slope	F-value
NINT	*Nitzschia intermedia*	0.512*	0.808	7.56**
SACU	*Synedra acus*	0.486*	1.236	16.12**
NREC	*Nitzschia recta*	0.476*	1.273	6.91*
DTEN	*Diatoma tenue*	0.438*	0.754	41.83***
NLIN	*Nitzschia linearis*	0.41**	1.199	41.79***

2.2 Data analysis

Physicochemical and biological databases from NOVANA and OMNIDIA, respectively, from the period 2013 to 2020, were combined by sampling site and year (n= 1036 sampling sites/time). The sites were distributed throughout Denmark and thus integrated different regions with different geology and land uses (Fig. 1).

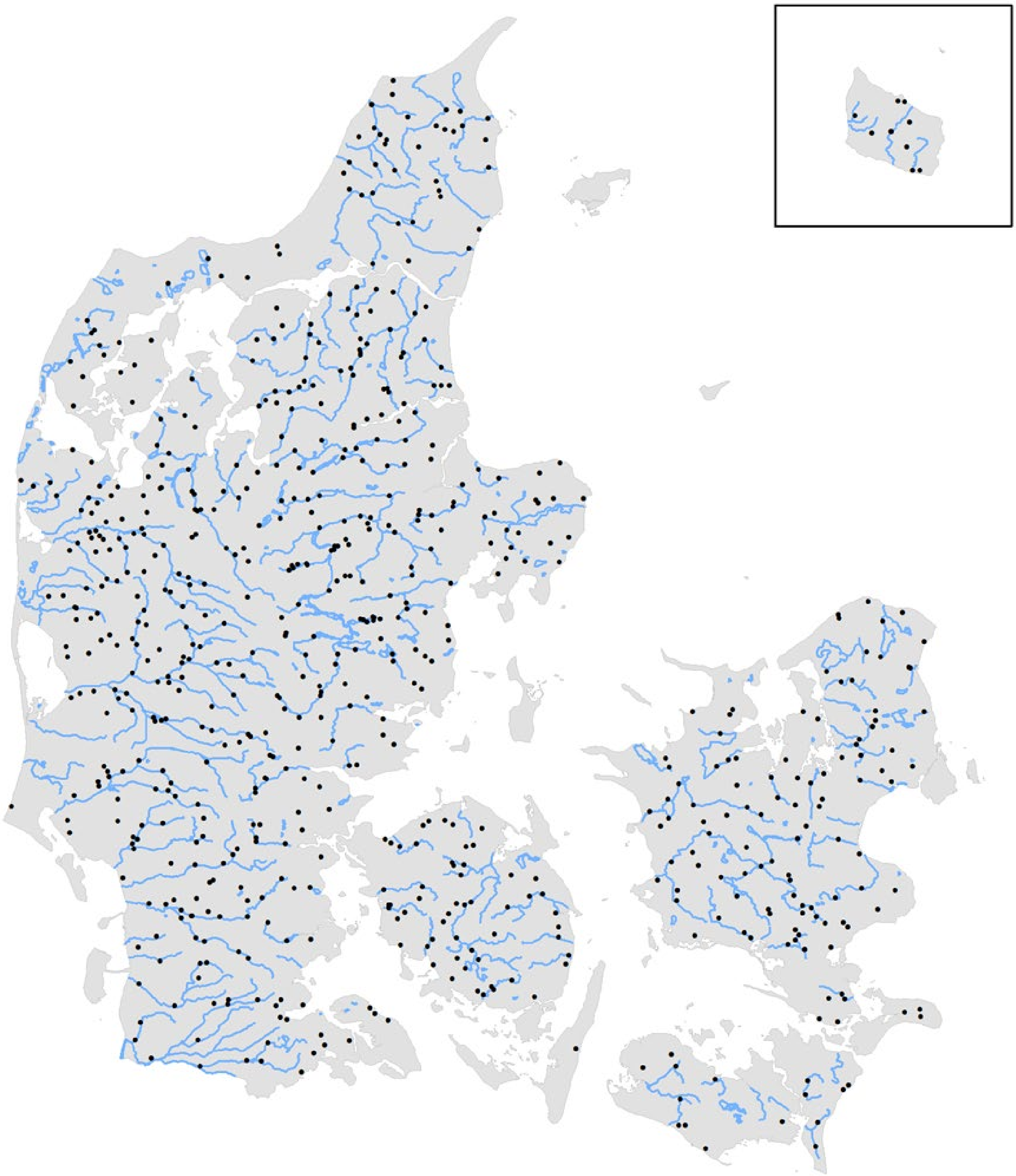

Figure 1. Location of the studied watercourses in Denmark for the period 2013-2020 (total n= 1036 sample sites / times). All sampling points included physicochemical and benthic diatom data.

Physicochemical and biological data were provided by the Danish Environmental Protection Agency/Miljøstyrelsen from Overfladevanddatabasen (ODA) and cross-checked with the data available from the VANDA database (https://vanda.miljoeportal.dk/). Previously analysed databases from 2013-2016 (Andersen *et al.,* 2018) were included and complemented with data for the period 2017-2020. Since physicochemical data are usually highly variable over time and differ markedly between warmer and colder months, the average physicochemical data for the April-September period (warmer months) of each year and sample station were considered. Stations/years with missing data for the analysed variables were excluded as well as data that were not quality assured by Miljøstyrelsen following the standard guidelines for NOVANA data treatment (https://ecos.au.dk/forskningraadgivning/fagdatacentre/ferskvand). For cross-analyses of physicochemical and biological data, sites without biological data available for the corresponding year were not considered.

The biological data from diatom quantification corresponded to the period 1 May to 15 May from 2013 to 2020 at the NOVANA control monitoring stations following the technical guidelines by the Danish Environmental Protection Agency/Miljøstyrelsen. These consist of standardised data based on the identification of diatoms to species level and the counting of 400 valves from each sample under direct microscope and included SID_TID values for each sample. Samples for counting are cleaned of organic material and mounted on slides, and the counting is performed by the same technician to avoid bias due to different species determination (Andersen *et al.*, 2018).

Physicochemical and biological variable distributions were initially graphically checked as to the frequency of value distribution, and outliers were identified from boxplots and removed by calculating the interquartile range (IQR) and removing values from the lower and upper limit of distribution following: Q1 – 1.5 * IQR (lower outliers) and Q3 + 1.5 * IQR Q1 (upper outliers).

The correlation between alkalinity and the variables related to eutrophication PO_4 and biological oxygen consumption (BI5, an indicator of biodegradable organic matter) was analysed as well as the correlation between SID_TID and alkalinity and PO_4. Likewise, it was analysed how the five diatom species previously identified to be indicative of high alkalinity were related to alkalinity and PO_4. In all cases, linear models assumed a confidence interval of 95% (p-value < 0.05) and normality and homoscedasticity on residuals distribution was checked. All statistical analysis were performed in R (R Core team 2022). Subsequently, the probability of fulfilment of the SID_TID values along the alkalinity gradient, was calculated using the EQR_SID_TID, that is, the EQR (Ecological Quality Ratio) index = observed index value/index reference value, for SID_TID:

EQR_SID_TID = (4 - Observed SID_TID) / (4 - Expected SID_TID)

where 4 is the maximum value that SID_TID can assume and the Expected SID_TID = 1,798, an average value from the unaffected or minimally affected watercourses, based on empirical data for Danish watercourses previously defined by Andersen *et al.* (2018). This probability model was expressed in EQR since this better reflects the ecological status compared to the Expected SID_TID reference quality value for these watercourses (Andersen *et al.,* 2018).

The probability of achieving target fulfilment requiring a SID_TID lower than 2.39 (limit between good ecological status and moderate ecological status) was expressed as probability for percentiles <5, 25, 50, 75 and >95%. The relationship between the EQR value and the log-transformed alkalinity was estimated as:

$$EQR\_SID\_TID = \alpha + \beta \cdot \log(Alk) + \varepsilon$$

where $\hat{\alpha}$ =0.85 (SE=0.0056), $\hat{\beta}$ =-0.094 (SE=0.0047), $\varepsilon \sim N(0,\sigma^2)$ and R^2=0.28. We assumed that EQR_SID_TID is distributed as a Gaussian stochastic variable with mean $\hat{\alpha} + \hat{\beta} \cdot \log(Alk)$ and variance σ^2= 0.015, which is the Mean Squared Error in the regression analysis. The probability of fulfilment of good ecological status for a given alkalinity value was calculated as:

P(EQR_SID_TID>k) = 1-P(EQR_SID_TID≤k)

where k is the limit value (0.73), and P is interpreted as the distribution function for the mentioned Gaussian distribution. The probability model was conducted using SAS version 9.4 (SAS Institute Inc).

For the literature review of the co-variation of alkalinity and PO_4 in freshwaters and ecological responses of target diatom species to alkalinity, a search for academic documents in English in the ISI web of knowledge (www.webofscience.com) and in Scholar Google with the following searching keywords and chains was conducted:

- Phosphate AND alkalinity AND water
- Orthophosphate AND alkalinity AND water
- Phosphorus AND alkalinity AND water
- PO_4 AND alkalinity AND water

For target diatom species, species names and the genus of each species listed in Table 1 along with alkalinity were searched following:

- (Species name) AND alkalinity
- Alkaliphilic AND (Species name) OR diatom

For the search by species name, previous names or synonyms were also considered and these alternative names were checked in AlgaBase online database search (https://www.algaebase.org/search/species/).

3 Results

3.1 Physicochemical and biological variables

Physicochemical data from sampling points distributed throughout Denmark (Fig. 1) were highly variable, ranging from low levels of alkalinity, nutrients in water and BI5 to relatively high levels (Table 2). The size of basins was also highly variable, ranging from small headwater basins to large basins (a spring in Østersø, Sealland of 0.02 km² to Skjern Å in Lønborg bro, 2315.39 km²).

The average of the SID_TID was 2.32, close to the value 2.39 being the limit for good ecological conditions (Table 2). Diatom species previously identified as indicating high alkalinity regardless of PO_4 were low in abundance, generally with less than one individual per sample, with especially low abundances for *Nitzschia intermedia* (NINT), *Nitzschia recta* (NREC) and *Synedra acus* (SACU) (Table 2).

Table 2. Physicochemical and biological variables from Overfladevanddatabasen (ODA) and OMNIDIA, respectively, for the studied watercourses in Denmark. Average, median, minimum, maximum and standard deviation (Std. Dev.) values for data from May to September from 2013 to 2020.

Physicochemical variables

	Alkalinity	**Ammonia + ammonium**	**Nitrite + nitrate**	**Total nitrogen**	**Ortho-phosphate**	**Total phosphorus**	**BI5**	**Catchment area**
		$NH_4^+ + NH_3$	$NO_2^- + NO_3^-$	TN	PO_4^{3-}	TP		
	(mEq/L)	(mg/L)	(mg/L)	(mg/L)	(mg/L)	(mg/L)	(mg/L)	(km²)
Average	3.07	0.117	2.846	3.195	0.069	0.123	1.30	64.12
Median	2.90	0.054	2.225	2.629	0.041	0.093	1.15	18.63
Minimum	0.11	0.003	0.009	0.140	0.002	0.006	0.25	0.02
Maximum	14.0	11.0	15.0	31.0	0.97	1.812	5.72	2315.39
Std. Dev.	1.75	0.289	2.232	2.234	0.075	0.211	0.72	167.37

Biological variables

	SID_TID	**Species number**	**Shannon's diversity**	**Evenness**	**NINT**	**SACU**	**NREC**	**DTEN**	**NLIN**	**Total abundance**
Average	2.32	31	3.24	0.66	0.08	0.97	0.40	2.46	1.00	5.2
Median	2.34	30	3.24	0.68	0	0	0	0	0	2.0
Minimum	0.96	12	0.88	0.17	0	0	0	0	0	0
Maximum	2.97	60	5.10	0.89	11.0	40.0	15.0	151.0	113.0	166.0
Std. Dev.	0.32	8.6	0.70	0.11	0.6	3.4	1.3	10.0	4.8	12.7

The physicochemical variables that indicate eutrophication, PO_4 and BI5, were positively correlated with alkalinity (Fig. 3.1, table 3). Although both models were significant, the adjustment correlation (adjusted R^2) of the alkalinity was higher for PO_4 than for BI5 (R^2 = 0.41 and 0.19 respectively, Table 3).

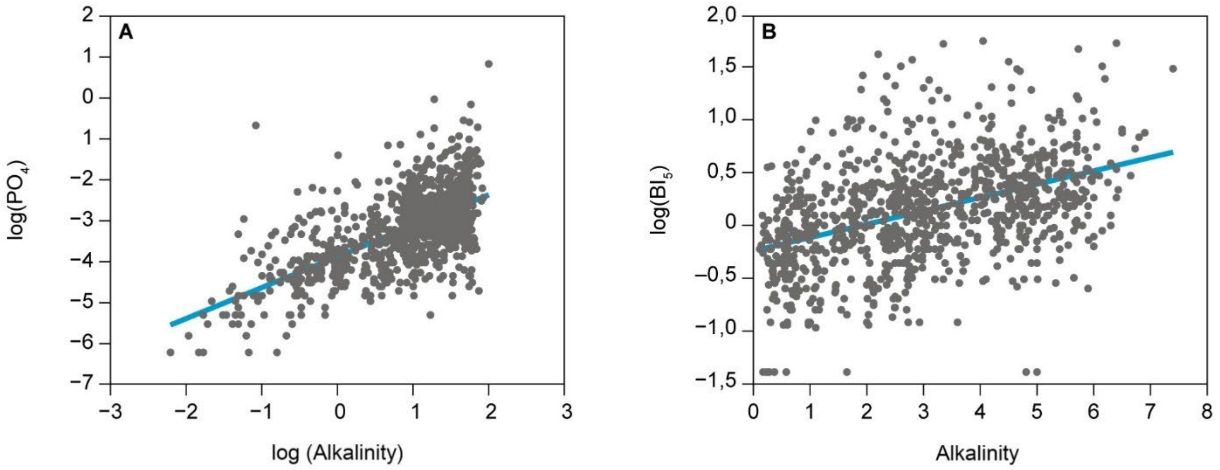

Figure 2. Linear correlation of orthophosphate (PO_4) and biological oxygen consumption (BI5) with alkalinity. Further details of the linear models are given in table 3.

Table 3. Linear correlation models of orthophosphate (PO_4) and BI5 in response to alkalinity. Equation used, results formula, adjusted R^2, F-value (F), p-value (p) and degrees of freedom (DF).

Equation	**Formula**	**Adjusted R^2**	**F**	**p**	**DF**
lm ($logPO_4$ ~ log Alk)	$logPO_4$= -3.876 + 0.756 * log(Alkalinity)	0.41	696.5	<0.001	1016
lm (log BI5 ~ Alk)	logBI5= -0.242 + 0.126 * Alkalinity	0.19	230.7	<0.001	999

3.2 SID_TID responses to alkalinity and orthophosphate

SID_TID was positively correlated with alkalinity as well as with orthophosphate (Fig. 3, Table 4). The correlation between SID_TID and alkalinity was higher (R^2=0.28) than between SID_TID and PO_4 (R^2=0.18, Table 4). Many SID_TID values were above the 2.39 limit of good ecological quality at intermediate PO_4 values but especially at intermediate to high alkalinity values (Fig. 3).

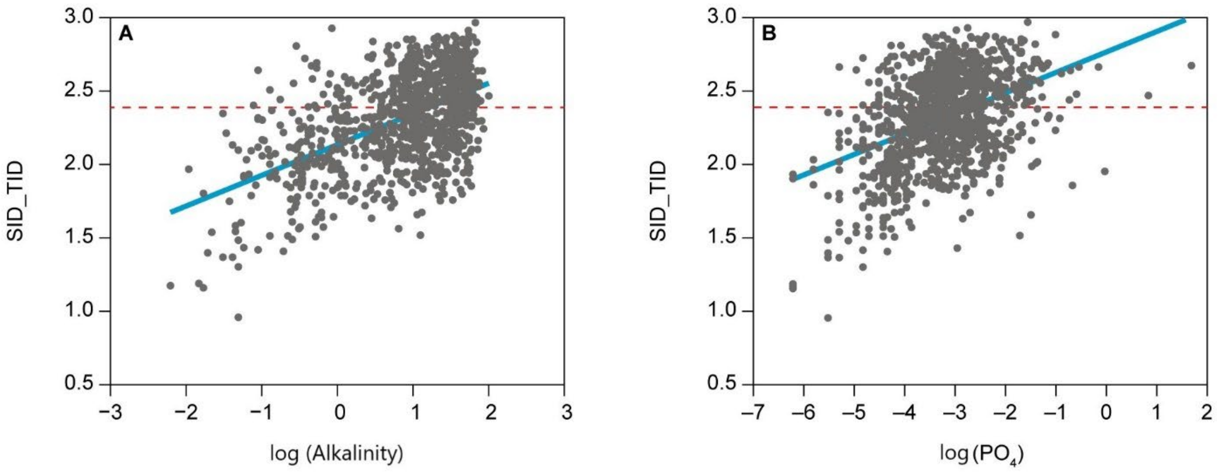

Figure 3. Linear correlation of SID_TID with alkalinity and orthophosphate (PO_4). Red dashed line indicates SID_TID= 2.39, upper limit of good ecological conditions. Further details of linear models are given in table 4.

Table 4. Linear correlation models for SID_TID in response to alkalinity and orthophosphate (PO_4). Equation used, results formula, adjusted R^2, F-value (F), p-value (p), and degrees of freedom (DF).

Equation	Formula	Adjusted R^2	F	p	DF
lm (SID_TID ~ log Alk)	SID_TID= 2.138 + 0.480 * log(Alkalinity)	0.28	394.9	<0.001	1016
lm (SID_TID ~ log PO_4)	SID_TID= 2.775 + 0.142 * log(PO_4)	0.18	223.4	<0.001	1016

3.3 Responses of target diatom species to alkalinity and PO_4

The linear model of the five diatom species identified in a previous study as indicators of high alkalinity regardless of PO_4 values (Table 1) showed that these were only poorly related to alkalinity in the present study (Fig. 4, Table 5). These species and their total abundance (sum of abundances) were even only marginally correlated to alkalinity and PO_4 ($R^2 < 0.03$; p value < 0.05) or not correlated (p value > 0.05; Fig. 4 and 5, Table 5).

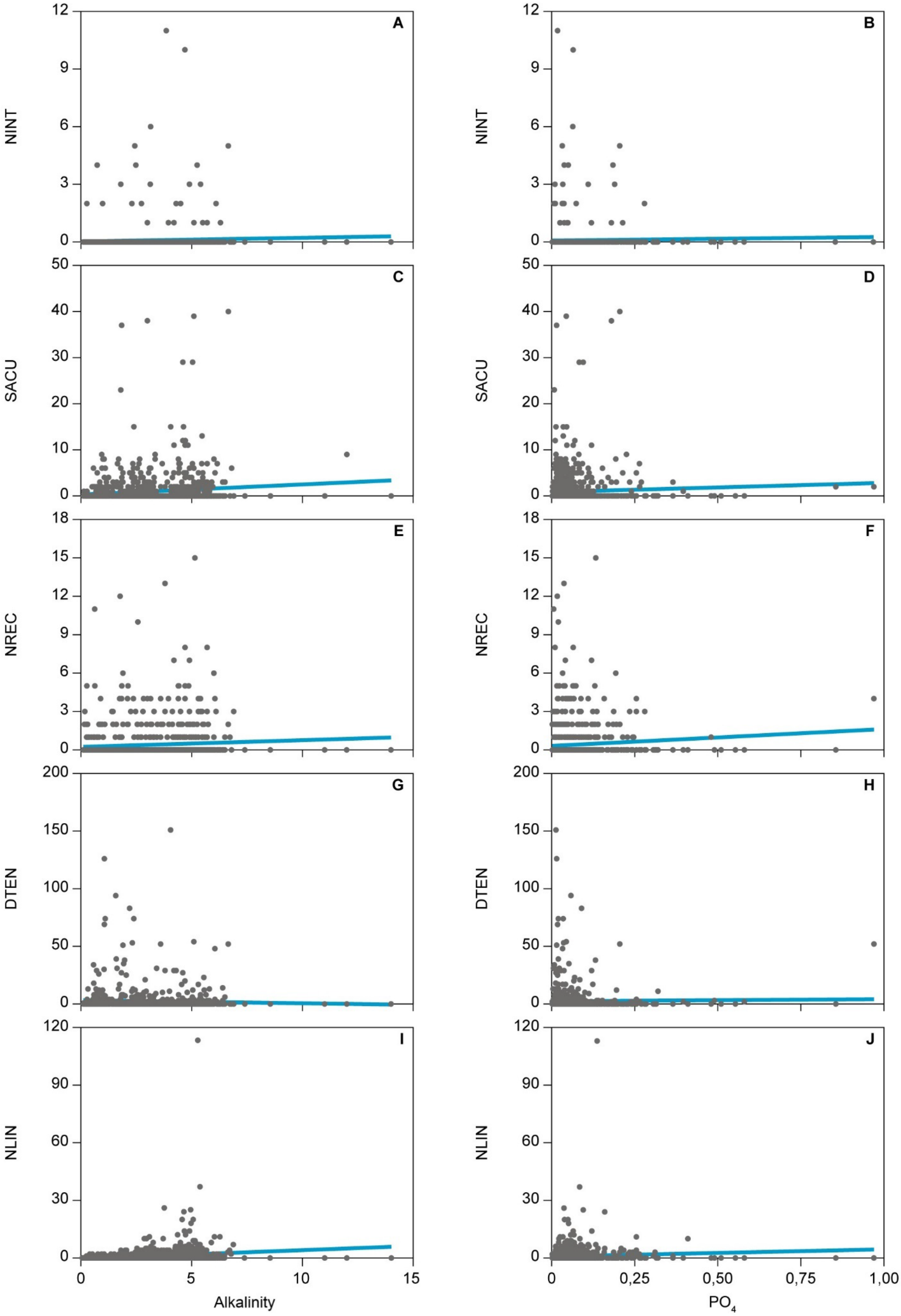

Figure 4. Linear correlation of target diatom species with alkalinity and orthophosphate (PO_4). Species names: *Nitzschia intermedia* (NINT), *Synedra acus* (SACU), *Nitzschia recta* (NREC), *Diatoma tenue* (DTEN), *Nitzschia linearis* (NLIN). Further details of linear models are given in Table 5.

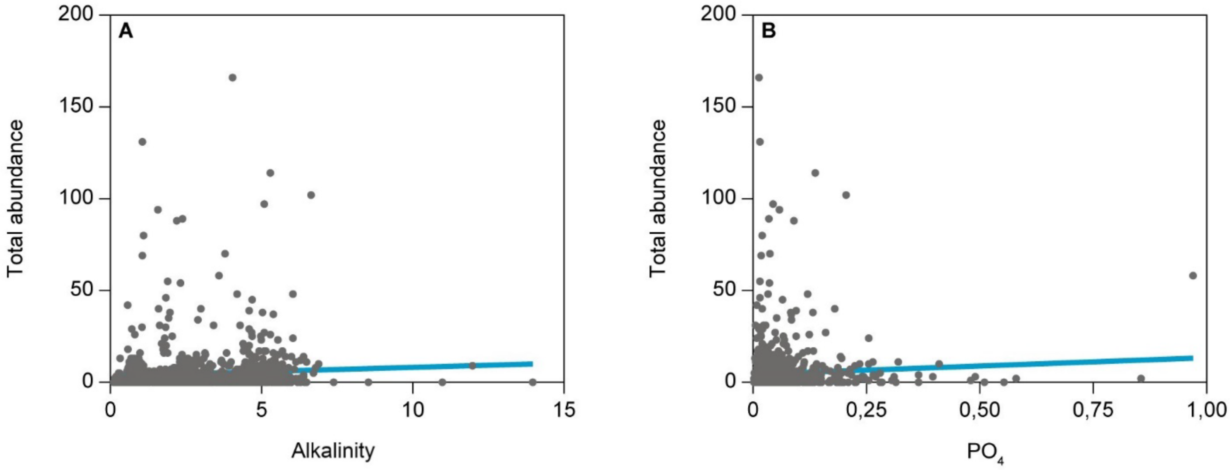

Figure 5. Linear correlation of the total abundance of target diatom species with alkalinity and orthophosphate (PO_4). Further details of linear models are given in table 5.

Table 5. Linear correlation models for target diatom species in response to alkalinity and orthophosphate (PO_4). Equation used, results formula, adjusted R^2, F-value (F), p-value (p), and degrees of freedom (DF). Species names: *Nitzschia intermedia* (NINT), *Synedra acus* (SACU), *Nitzschia recta* (NREC), *Diatoma tenue* (DTEN), *Nitzschia linearis* (NLIN). Total: sum of the target species abundances.

Equation	Formula	Adjusted R^2	F	p	DF
lm (NINT ~ Alk)	NINT= 0.024 + 0.0190 * Alkalinity	0.002	2.80	0.09	1020
lm (SACU ~ Alk)	SACU= 0.301 + 0.219 * Alkalinity	0.012	13.87	<0.001	1020
lm (NREC ~ Alk)	NREC= 0.238 + 0.052 * Alkalinity	0.004	5.02	0.022	1020
lm (DTEN ~ Alk)	DTEN= 3.267 - 0.267 * Alkalinity	0.001	2.28	0.131	1020
lm (NLIN ~ Alk)	NLIN= -0.34 + 0.439 * Alkalinity	0.030	32.84	<0.001	1020
lm (Total ~ Alk)	Total= 3.490 + 0.461 * Alkalinity	0.003	4.33	0.038	1020
lm (NINT ~ PO_4)	NINT= 0.070 + 0.185 * PO_4	-0.001	0.48	0.488	1020
lm (SACU ~ PO_4)	SACU= 0.847 + 1.987 * PO_4	0.001	2.03	0.155	1020
lm (NREC ~ PO_4)	NREC= 0.317 + 1.311 * PO_4	0.005	5.88	0.015	1020
lm (DTEN ~ PO_4)	DTEN= 2.347 + 1.801 * PO_4	0.001	0.19	0.667	1020
lm (NLIN ~ PO_4)	NLIN= 0.767 + 3.721 * PO_4	0.003	4.12	0.043	1020
lm (Total ~ PO_4)	Total= 4.348 + 9.006 * PO_4	0.002	2.96	0.086	1020

3.4 Probability model of SID_TID along an alkalinity gradient

Since it was not possible to use the abundance of diatom species to disentangle the influence of alkalinity from that of PO_4 on SID_TID values, another approach was considered. Instead, the relationship between alkalinity and SID_TID was applied as a mean to identify the level of alkalinity that may jeopardise an assessment of eutrophication by using SID_TID in the watercourses. Based on the linear relationship of SID_TID as a function of alkalinity (Fig 3a and Table 4), probability-based pressure-response curves were generated to predict the probability of achieving minimum good ecological status based on values of SID_TID < 2.39 (limit of good ecological status in watercourses). The probability model expressed in EQR_SID_TID (see methods) indicates that watercourses with an alkalinity lower than 2.6 mEq/L have high probabilities (>95%) of fulfilment of SID_TID (< 2.39; good ecological status, Figure 6 and Table 6), whereas watercourses with an alkalinity higher than 2.6 mEq/L have a declining probability of fulfilment of SID_TID, within a short range of alkalinity (Figure 6 and Table 6). These thresholds correspond to the co-variation with PO_4 and are therefore not the direct result of causal relationships exclusively attributable to alkalinity.

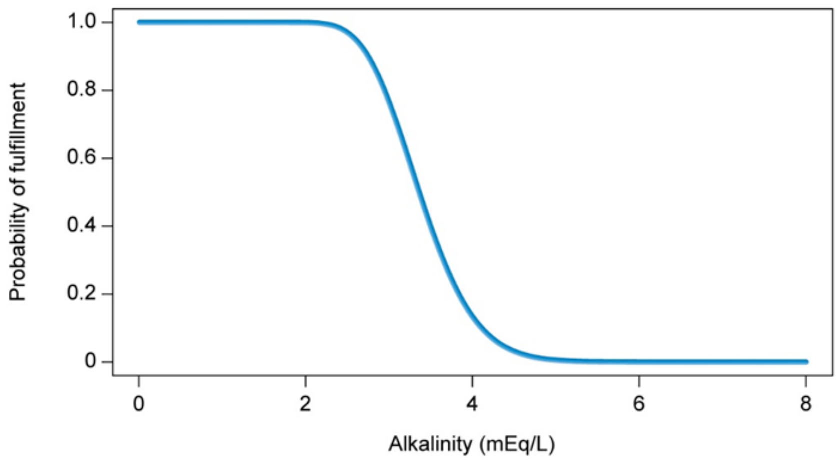

Figure 6. Probability model of good ecological status fulfilment (SID_TID < 2.39) along an alkalinity gradient in Danish running waters based on the Expected Quality Reference for SID_TID index (EQR_SID_TID). Model: R^2 = 0.28, F= 400.92, p-value < 0.001, DF= 1022. Probabilities of target fulfilment for different confidence percentages are expressed in Table 6.

Specifically, alkalinity thresholds for the probability of meeting good ecological status were calculated using EQR_SID_TID applying < 5%, 25%, 50%, 75% and > 95% as probability levels translating into an alkalinity of 4.4 mEq/L, 3.7 mEq/L, 3.4 mEq/L, 3.0 mEq/L and 2.6 mEq/L, respectively (Table 6). Interpretations based on this model should consider that the base linear model used to generate these probabilities was significant ($p < 0.01$) but also that the fraction of the variance explained (R^2) was restricted to 28% (Table 4).

Table 6. Probabilities of good ecological status fulfilment for different alkalinity values in Danish running waters based on the Expected Quality Reference for SID_TID index (EQR_SID_TID). The table shows at which alkalinity values there is a <5%, 25%, 50%, 75% and >95% probability of achieving good ecological status fulfilment according to SID_TID < 2.39.

Probability of target fulfilment	<5%	25%	50%	75%	>95%
Alkalinity value (mEq/L)	4.4	3.7	3.4	3.0	2.6

3.5 Literature review on the co-variation of alkalinity and PO_4 in freshwaters and ecological responses of target diatom species to alkalinity

The literature review of published papers on indexed databases showed several papers emphasising the co-variation of alkalinity and phosphorus (mainly as PO_4) in freshwaters (Table 7).

Table 7. Review of possible co-variation of alkalinity with orthophosphate in freshwaters, main evidence, and associated references. For further details on the searching criteria see methods section.

Evidence of alkalinity and orthophosphate relationship in freshwaters	Reference
Co-variation of alkalinity (natural origin) and PO_4 (human-induced) in Danish streams may cause failure to comply water quality standards. Alkalinity affects benthic diatom composition in lowland streams due to different efficiency of species in the use of HCO_3. These diatom species can be used as indicators of alkalinity regardless of PO_4 values.	Baattrup-Pedersen *et al.* (2022).
Alkalinity is generally attributable to HCO_3^-, CO_3^2 and OH^- but also to other components in smaller amounts such as silicates, borates, ammonia, phosphates (e.g., PO_4) and organic bases.	Sayilgan and Arol (2004)
Alkalinity is correlated with phosphorous, and these are the main influencing factors controlling phytoplankton biomass and composition in 1558 lakes in 20 countries of Europe. Alkalinity has a key role in determining phytoplankton species composition in European lakes. Alkalinity and phosphorous were strongly correlated with many cyanobacteria species.	Maileht *et al.* (2013)
Failure to comply with Water Framework Directive standards for good phosphorous status related to high alkalinity and phosphorous impairment in lowland streams and rivers. Lowland headwater streams and rivers can reach high phosphorous values only when alkalinity is high. In contrast, under low alkalinity conditions phosphorous values are always low.	Jarvie *et al.* (2018)
Alkalinity can come from different sources, including carbonate alkalinity and phosphate alkalinity, and this can affect biofilm nitrification activity.	Biesterfeld *et al.* (2003)
Alkalinity has largely increased in US as a consequence of chemical weathering, which is related to the process of salinisation of freshwaters and can be related to ions as PO_4. Agriculture, mining activities and urbanisation can influence the alkalinity by soil liming, acid mine drainage and runoff from impervious surfaces, respectively. The effects of urban land use in interaction with the watershed geology on alkalinity concentrations in streams and rivers warrant further study.	Kaushal *et al.* (2013)
Agricultural liming can be a dominant source of increasing carbonates in agricultural watersheds over the previous century, and this can increase alkalinity.	Raymond and Cole (2003) Raymond *et al.* (2008)
Changes in ion concentrations due to human activities as agriculture or mining can increase alkalinity in freshwaters. One of these ions is PO_4, which is found as H_2PO_4 and HPO_4 and HPO_4 in agricultural fertilizers.	Cañedo-Argüelles *et al.* (2016) Schlesinger (2021)
PO_4 can be related to alkalinity, since it can originate from natural processes such as weathering of sedimentary rocks (e.g., phosphate-bearing limestone, apatite minerals) but can also be increased by inorganic fertilisers, farm manure and animal waste.	Kaushal *et al.* (2021) Manning (2015) Meyer (1980)
Alkalinity is widely used as a predictor of chemical and biological water quality standards in rivers under the EU Water Framework Directive, but significant input of anthropogenic alkalinity to rivers from effluents and catchment runoff has the potential to undermine its usefulness, as illustrated by river phosphorous standards. Alkalinity is usually attributed to rock weathering and is free of anthropogenic influences. However, the potential contribution of anthropogenic alkalinity needs to be considered and quantified. Anthropogenic alkalinity is likely to be present in diffuse runoff, but it is difficult to apportion alkalinity loads between natural and contaminant sources.	Tappin *et al.* (2018)

Some of these studies indicated the relevance of these co-variating variables for algae and cyanobacteria (Meileht *et al.*, 2013; Biesterfeld *et al.*, 2003). Others indicated the role of alkalinity in failing to comply with the Water Framework

Directive due to this co-variation (Jarvie *et al.*, 2018; Tappin *et al.*, 2018). Many other recent papers have also indicated that while alkalinity is often considered as being almost exclusively of natural origin, there are other processes related to human activities that may increase the natural alkalinity (Table 7). In some cases, high alkalinities were explained by anthropogenic impacts that also increased PO_4 (Table 7).

Target diatom species (Table 1) have been associated with high alkalinity or mentioned as indicators of high alkalinity in different studies or reviews of different ecosystems and countries (Table 8).

Table 8. Review of the relationship of diatom species previously identified as indicators of high alkalinity regardless of PO_4. For further details on the searching criteria see methods section.

Species	Evidence of diatom species as indicators of alkalinity without relation to PO_4.	Reference
Nitzschia intermedia (NINT)	Described as an alkaliphilic species in Pantanal, Brazil.	Malone *et al.* (2012)
	In an alkaline lake in Turkey.	Sonmez *et al.* (2018)
Synedra acus (synonym *Ulnaria acus*) (SACU)	Related to low alkalinity (0.26 mEq/L) in Minnesota, US.	Brugam and Patterson (1983)
	At intermediate alkalinity (2.30 mEq/L), in Kenya.	Lind *et al.* (1968)
	Indicator of high alkalinity in alkaline River Donghe, China.	Liu *et al.* (2019)
	Associated with increased alkalinity in temporal wetlands, Brazil.	Riato *et al.* (2017)
Nitzschia recta (NREC)	Indicator of high alkalinity. Review.	Van Dam *et al.* (1994)
	Associated with high alkalinity in hot springs, Kenya.	Owen *et al.* (2008)
	Associated with high alkalinity in urban tropical stream, Kenya.	Ndiritu *et al.* (2006)
Diatoma tenue *Diatoma tenuis* (DTEN)	Indicator of high alkalinity. Review.	Van Dam *et al.* (1994)
	Associated with high alkalinity in upland peat forest rivers, Ireland.	O´Driscoll *et al.* (2012)
Nitzschia linearis (NLIN)	Indicator of high alkalinity. Review.	Sonmez *et al.* (2018) Van Dam *et al.* (1994)
	Associated with high alkalinity in Pinal Creek, US.	Spindler *et al.* (1996)
	High alkalinity, large rivers in the US.	Fore and Grafe (2002)

4 Discussion

Physicochemical parameters were highly variable despite being temporally integrated as average data for the same months (from April to September). This indicates that besides the temporal variability, there is a wide range of spatial variability in these parameters among Danish watercourses. The high average values of (SID_TID = 2.32) close to the limit of good ecological quality (SID_TID < 2.39) indicate that many Danish watercourses are close to the limit or even exceeded the limit for good ecological status (Table 2, Figure 3).

Alkalinity was positively correlated with PO_4 and BI5, both variables being related to eutrophication (Table 3, Figure 2), and the correlation was higher for PO_4 than for BI5. This indicates that alkalinity is more associated with nutrient levels in water, and in particular with PO_4, than with organic matter content. These results support the co-variation of alkalinity and PO_4 for Danish watercourses as previously described (Baattrup-Pedersen *et al.*, 2021, 2022) but also its correlation to BI5 as an estimator of organic matter load. Multiple studies support these results and associate alkalinisation with the process of salinisation and eutrophication (Table 7). Although alkalinity has classically been considered to be mainly naturally influenced by the geology of the catchments, there is much recent evidence supporting that various human activities can foster the two processes together, e.g., agriculture and urbanisation (Raymond and Cole, 2003; Kaushal *et al.*, 2013, 2021).

When comparing the relationship of the ecological index SID_TID with PO_4 and alkalinity, surprisingly SID_TID was more related to alkalinity than to PO_4 (Figure 3, Table 4). These results also support previous findings of the influence of alkalinity on estimations of ecological status based on SID_TID (Baattrup-Pedersen *et al.*, 2021, 2022). Moreover, these results reaffirm the critical role of alkalinity in influencing SID_TID. The strong association between nutrient pollution and high alkalinity and the closer response of SID_TID to alkalinity than to PO_4 are an interesting topic to be further considered and investigated, especially the spatial and temporal variation of these factors and the potential causes of this co-variation.

In this study, the possibility to disentangle the role of alkalinity from PO_4 when applying SID_TID was explored by using target species previously identified as indicators of high alkalinity and low PO_4 (Baattrup-Pedersen *et al.*, 2022). These species were also identified by previous authors to be associated with high alkalinity in different inland waters worldwide (Table 8). This association with high alkalinity has been attributed to differential access and use of the carbon sources that are affected by alkalinity and in particular with a more efficient use of bicarbonate (HCO_3^-) being increasingly available when alkalinity increases (Stumm and Morgan, 1995; Clement *et al.*, 2017). However, contrary to our expectations, no evidence of any relationship of these target species with either alkalinity or PO_4 could be found, and, furthermore, these species were not found to be particularly abundant at high alkalinities (Figure 4, Table 2, Table 5). This can be attributable to different factors. The target species were not registered in many watercourses, which makes these species difficult to compare among ecosystems. Furthermore, these species were identified as indicator species under very particular conditions: experimental substrates at high alkalinity and low PO_4 (Baattrup-Pedersen *et al.*, 2022). The combination of high alkalinity and low PO_4 is not typical for Danish watercourses, and species responses may, therefore, be different under these conditions. Thus, the rather high abundance of these species in the experiment where they were associated with high alkalinity and low PO_4 can be related to the experimental factors (e.g., new substrate without previous colonisation, short successional times, etc.) that may have created a particular window of opportunity for these species.

Since previously identified target species were not adequate proxies to disentangle the co-variation between alkalinity and PO_4 in influencing SID_TID, it was decided to develop probability models based on alkalinity values. The probability models indicated that alkalinity values greater than 2.6 mEq/L were associated with a rapid decrease in the probability of compliance with good ecological status (SID_TID < 2.39; Figure 6, Table 6). The probability of fulfilment of target values for good ecological conditions for SID_TID was higher than 95% at alkalinity values lower than 2.6 mEq/L and lower than 5% at values higher than 4.4 mEq/L. This can be considered as a short range of variation and may be due to the relatively strong correlation of SID_TID with alkalinity (Figure 3, Table 4). The calculated probabilities may form the basis for the selection of watercourses for ecological assessment based on SID_TID. Depending on the alkalinity levels, watercourses can be selected based on the risk for not meeting good ecological targets evaluated by SID_TID at different probabilities (Fig. 6, Table 6).

The present results suggest that alkalinity can have a stronger influence on SID_TID than PO_4 under natural conditions in Danish watercourses, and furthermore that alkalinity values being higher than 2.6 mEq/L can be associated with reduced probability of fulfilling good ecological status (Figure 6, Table 6). In the literature review, it was found that many recent studies link this covariation of PO_4 and alkalinity to a combination of natural causes and anthropogenic impacts that may add to explain the frequent association of these two variables in inland waters (Table 7).

Alkalinity is usually attributed to natural characteristics such as catchment geology and rock weathering, but our findings together with the previously mentioned studies indicate that anthropogenic impacts may be influencing alkalinity in Danish running waters. In Denmark, high alkalinity has been largely perceived as a natural characteristic of catchments with high contents of lime in the underground (Rebsdsorf *et al.*,1991; Kronvang *et al.*,2006) and even as a positive characteristic since it decreases the risk of acidification (Rebsdsorf *et al.*, 1991). However, this perspective is nowadays challenged with recent data that sees to confirm a worldwide trend: agricultural land use and other human activities seem to increase the alkalinity of freshwaters (Table 7). Rebsdsorf *et al.* (1991) found an acidification trend in western Jutland streams from 1977 to 1989 and attributed this to fertilisation in heavily cultivated catchments. Recent studies found that in catchments characterised by high contents of lime in the underground and thus ample buffering capacity, acidification by agricultural practices (e.g., fertiliser usage, organic acids production, soil removal) mediated an increase in alkalinity (Van Breemen *et al.*, 1983; Amiotte Suchet *et al.*, 1995; Raymond and Cole, 2003; Kaushal *et al.*, 2013). Catchments with high contents of lime in the underground therefore seem to be more vulnerable to alkalinisation (Raymond and Cole 2003; Stets *et al.*, 2014), with carbon lithology being the main predictor variable for alkalinisation (Kaushal *et al.*, 2013). The causes behind alkalinisation are mainly attributed to urbanisation and agriculture. Urbanisation increases weathering from disturbed soils, concrete and calcium that degrade over time as extra weatherable material and elevated CO_2 due to sewer or septic effluents (Barnes and Raymond, 2009; Davies *et al.*, 2010; Kaushal *et al.*, 2012). Agriculture stimulates soil erosion and increase weathering, and along with fertiliser usage, acidification and agricultural liming with calcium carbonate ($CaCO_3$) or dolomite ($MgCa(CO_3)_2$) may increase water alkalinity, especially so in highly agricultural catchments (Raymond and Cole, 2003; Hamilton *et al.*, 2007; Barnes and Raymond, 2009; Aquilina *et al.*, 2012). These processes are further enhanced by increased precipitation and runoff (Raymond and Cole, 2003) and may therefore be intensified as the climate changes.

Alkalinisation have been reported in large rivers in the US (Raymond and Cole, 2003) where Stets *et al.* (2014) found a trend in alkalinisation in 14 out of 23 rivers along the US, and Kaushal *et al.* (2013) registered the same process in 50% of the western US rivers. Average alkalinity in Danish streams varied between 0.59 and 2.24 mEq/L moving from western to eastern Denmark back in 1977-1989 with maximum values of *ca*. 1.30 and 4.50 mEq/L for the two regions, respectively, to an average of 3.07 and maximum of 14.0 mEq/L in 2013-2020 (Table 2). This means an average increase of *ca*. 330% in alkalinity compared to 1977-1989 and *ca*. 700% in maximum values. However, there are still no published studies on alkalinisation in Danish watercourses, and the generality of the above-described trend therefore needs to be explored further. Some recent studies refer to the process of anthropogenic alkalinisation, particularly related to freshwater salinisation processes (Biesterfeld *et al.*, 2003; Cañedo-Argüelles *et al.*, 2016; Kaushal *et al.*, 2013; Kaushal *et al.*, 2021; Manning, 2015; Schlesinger, 2021). This is a relatively new emerging threat in freshwaters worldwide, which urges further research – especially due to its relevant potential consequences for freshwater ecosystems. For instance, Maileht *et al.*, (2013) found that along with PO_4, alkalinity was the main influencing factor explaining high phytoplankton biomass and compositional changes and that alkalinity was associated with many species of potentially toxic cyanobacteria in 1558 lakes in 20 countries in Europe. Some studies emphasise the potential role of alkalinisation in the global carbon cycle and in the increase of the carbon transport to marine ecosystems, thus inducing marine alkalinisation and changes in carbon saturation balances (Meybeck, 1987; Mackenzie *et al.*, 2011; Kaushal *et al.*, 2013). The potential interaction of anthropogenic causes with natural alkalinity – and therefore the contribution of anthropogenic alkalinity to the natural alkalinity – needs to be further considered and quantified at different temporal and spatial scales in Danish inland waters.

5 Conclusions

In the present it was found that alkalinity is directly coupled to PO_4 and therefore to SID_TID in Danish running waters and that SID_TID may be affected by alkalinity to such an extent that is not possible to use this index to assess the ecological status in response to PO_4 as a proxy of anthropogenic eutrophication. Surprisingly, the correlation of alkalinity with SID_TID was even higher than with PO_4, displaying that this association can strongly interfere with the possibility to achieve environmental targets in Danish watercourses by applying SID_TID.

Target benthic diatom species were not related to alkalinity, and the results do therefore not support previous findings that these species could potentially be used to disentangle the influence of alkalinity from PO_4 (Baattrup-Pedersen 2021, 2022). Instead, the empirical relationship between alkalinity and SID_TID was applied to derive the probability of reaching minimum good ecological status in watercourses along a gradient in alkalinity. The probability of reaching good ecological status (based on SID_TID < 2.39) exceed 95% in Danish watercourses with an alkalinity below 2.6 mEq/L and decline to 50% at an alkalinity of 3.4 mEq/L.

Due to the significant effect of alkalinity as a factor interfering with the assessment of ecological status in watercourses, and its association to indicators of eutrophication, the study of alkalinity, its sources, and its temporal and spatial variability together with geology and possible anthropic effects should be better explored in order to effectively manage watercourses.

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ALKALINITY AND ITS INFLUENCE ON BENTHIC DIATOM ASSESSMENTS IN DANISH RUNNING WATERS

Alkalinity and PO4 co-varies in Danish running waters, and this affects the applicability of the diatom-based indicator of eutrophication SID_TID. Through the analysis of physicochemical and diatom databases, we evaluated the influence of alkalinity on the ability to meet ecological targets and sought to disentangle the effect of alkalinity by the abundance of target diatom species as indicator of high alkalinity and low PO_4. Alkalinity was correlated to trophic status indicators (PO_4 and BI5), confirming that it is an important interfering factor influencing the SID_TID, and we thus discuss potential causes of the association between these factors. We found no relationship of alkalinity with the abundance of target diatom species. We then developed a probability model, which indicated that at alkalinity values greater than 2,6 mEq/L the probability of meeting ecological targets based on the SID_TID declines drastically and, therefore, the convenience of this index at higher alkalinities should be evaluated.

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