Elevated alkalinity and sulfate adversely affect the aquatic macrophyte *Lobelia dortmanna*

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Abstract The increase in alkalinity and SO_4^{2-} in softwater lakes can negatively affect pristine isoetid population because the increase in alkalinity and SO_4^{2-} can stimulate sediment mineralization and consequently cause anoxia. The consequences of increased sediment mineralization depend on the ability of isoetids such as *Lobelia dortmanna* to oxidize the rhizosphere via radial O_2 loss. To study how alkalinity and SO_4^{2-} affect the isoetid *L. dortmanna*, and if negative effects could be alleviated by neighboring plants, three densities of *L. dortmanna* ("Low" = 64 plants m^{-2}, "Medium" = 256 plants m^{-2} and "High" = 1,024 plants m^{-2}) were exposed to elevated alkalinity in the water column, or a combination of both elevated alkalinity and SO_4^{2-}, and compared to a control situation. The combination of SO_4^{2-} and alkalinity significantly increased mortality, lowered areal biomass and reduced actual photosynthetic efficiency. Plant density did not significantly alleviate the negative effects caused by SO_4^{2-} and alkalinity. However, actual photosynthetic efficiency was significantly positively correlated to redox potential in the sediment, indicating a positive relationship between plant performance and sediment oxidation. The negative effects on *L. dortmanna* were probably caused by long periods of tissue anoxia by itself or in combination with H_2S intrusion. Therefore, increase in both SO_4^{2-} and alkalinity surface water can dramatically affect *L. dortmanna* populations, causing reduction or even disappearance of this icon species.

Keywords Isoetid · Redox potential · Sulfide · Softwater lake · *Lobelia dortmanna* · Photosynthetic efficiency

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Introduction

Softwater lakes have low surface water alkalinity (<1 mM) and are naturally inhabited by slow-growing aquatic macrophytes called isoetids that are adapted to carbon and nutrient-limited systems (Arts 2002;

Murphy 2002). Increasing alkalinity (alkalinization) above the low natural levels is among the main causes of degradation of the vegetation in softwater lakes (Arts 2002). Alkalinization happens by direct lime application used as a restoration measure to revert acidification (Brandrud 2002), by hydrological changes such as the introduction of alkaline water (Smolders and Roelofs 1995) or internally due to in-lake processes such as the reduction in SO_4^{2-}, NO_3^- or Fe in sediment mineralization processes (Psenner 1988; Smolders et al. 2006). Alkalinization can cause a shift in the vegetation from plant species character-istic of softwater (isoetids) to species characteristic of buffered conditions (elodeids, and other hard-water macrophytes) (Arts 2002). Elodeids benefit from the higher inorganic carbon availability and are fast-growing, tall and ramifying plants that eventually overgrow the smaller slow-growing isoetids (Roelofs et al. 1994; Brandrud 2002; Lucassen et al. 2009; Spierenburg et al. 2009; 2010).

In addition to alkalinization, SO_4^{2-} has also been reported to increase above the naturally low levels as a consequence of burning of fossil fuels. However, high SO_4^{2-} concentrations alone do not seem to impact the natural vegetation as concentrations above 1 mM have been reported in pristine isoetid lakes (Roelofs 1983). In contrast, it has been observed that the combined high SO_4^{2-} and alkalinity result in regression or even total disappearance of isoetids (Brouwer et al. 1999). The negative effects produced by the combination of high SO_4^{2-} and alkalinity seem to be linked to sediment mineralization processes (Brouwer et al. 1999). In anoxic sediments, mineralization is stimu-lated by SO_4^{2-}, which acts as an alternative electron acceptor to O_2 (Ponnamperuma 1984; Laanbroek 1990), and by alkalinity, which neutralizes decay-inhibiting acids and thus creates a more suitable environment for decomposer organisms (Traaen 1980; Kok and Van der Velde 1991). Increasing sediment mineralization leads to higher sediment O_2 demand and results in reduced conditions. Although isoetids can resist short periods of tissue anoxia (Møller and Sand-Jensen 2011), long periods of tissue hypoxia and/or anoxia may decrease vascular translocation (Sorrell 2004), photosynthetic efficiency and plant growth (Pulido and Borum 2010) and can even cause mortality of sensitive aquatic macrophytes (Sand-Jensen et al. 2005a; Raun et al. 2010). Moreover, under reduced conditions, SO_4^{2-} is converted to

sulfide which is known to be highly phytotoxic (Koch and Mendelssohn 1989; Goodman et al. 1995; Holmer and Bondgaard 2001; Geurts et al. 2009).

On the other hand, elevated sediment mineraliza-tion can also benefit the isoetid vegetation if the plants are able to maintain the rhizosphere oxidized (Pulido and Borum 2010; Pulido et al. 2011). Isoetids have a high radial O_2 loss across the entire root system (Sand-Jensen et al. 1982; Pedersen et al. 1995), and as a result, the rhizosphere is often permanently oxic, and problems with reduced phytotoxins are thus prevented (Holmer et al. 1998; Pedersen et al. 2004). Under such conditions with an oxic rhizosphere, the increased production of CO_2, linked to sediment mineralization (Laanbroek 1990; Mattson and Likens 1993), can stimulate the underwater photosynthesis (Søndergaard and Sand-Jensen 1979; Roelofs et al. 1984; Pedersen et al. 1995) and growth of the isoetids (Madsen et al. 2002; Andersen et al. 2005; Pulido et al. 2011). Therefore, the overall outcome of increased sediment mineralization depends on the ability of isoetids to oxidize their rhizosphere (Pulido et al. 2011), and an effective oxidation of the rhizosphere partly depends on plant density (Tessenow and Baynes 1978). While a solitary plant might have difficulties to maintain an oxic rhizosphere, a dense mat of plants could build up a high O_2 bulk during the day and thereby prevent anoxic conditions overnight (Tessenow and Baynes 1978).

In the present investigation, our aim was to test whether alkalinity and SO_4^{2-} in surface water can directly affect the isoetid L. dortmanna, and whether potential impacts can be counteracted by higher plant density. Therefore, we tested the interactive effect of three surface water qualities (1) control, (2) high alkalinity and (3) a combination of high alkalinity and SO_4^{2-} at three plant densities ("Low" = 64 plants m^{-2}, "Medium" = 256 plants m^{-2} and "High" = 1,024 plants m^{-2}) on L. dortmanna. We hypothesized that (a) alkaline water effects on plant performance would depend on the balance between the positive (high CO_2 availability) and the negative (reduced conditions) effects related to an increase in sediment mineralization; (b) the combination of high alkalinity and SO_4^{2-} would negatively affect the plants, since they might be exposed to multiple stressors such as reduced sediment conditions and phytotoxicity; and (c) high plant density would counteract the negative effects by oxidation of the rhizosphere.

Materials and methods

Experimental design

A multifactorial experiment was designed to study the effects of alkalinity and SO_4^{2-} on *L. dortmanna* growing at three densities: "Low" (64 plants m^{-2}), "Medium" (256 plants m^{-2}) and "High" (1,024 plants m^{-2}). These densities were exposed to three water treatments: "Control" (3 mM NaCl; to keep the ion level similar to "Alk" and "SO_4^{2-} + Alk"), "Alk" (1 mM Ca(HCO$_3$)$_2$ and 2 mM NaCl) and "SO_4^{2-} + Alk" (1 mM Ca(HCO$_3$)$_2$, 1 mM Na$_2$SO$_4$, 1 mM NaCl). The concentrations used are characteristic for local groundwater and river water in The Netherlands (Smolders et al. 2006). Each treatment was randomly assigned to an aquarium (see below) and replicated four times.

Thirty-six aquaria (length = 12.5 cm, width = 12.5 cm, height = 31 cm) were placed in a water jacket and maintained at 20 ± 1 °C by means of a recirculation chiller (Neslab Merlin M-75; Thermo Scientific, Newington, NH, USA). Irradiance was 200 μmol m^{-2} s^{-1} and was provided by six Philips-type HP 400-W lamps (Hortilux-Schréder, Monster, The Netherlands) at a photoperiod of 12 h. The aquaria were filled with 15 cm (2.5 L) of fresh oligotrophic poorly buffered sediment collected from the softwater lake Banen (The Netherlands, 51°16′09 N, 05°48′05 E). Banen sediment had 48.6 ± 0.9 % of water, a density of 1.35 ± 0.05 kg L^{-1} (fresh sediment), and contained 10.3 ± 0.2 % of organic matter (dry mass). For the fresh Banen sediment, plant available P (Olsen et al. 1954) was 43.4 ± 27.0 μM, plant available NO$_3^-$ and NH$_4^+$ was 58.9 ± 1.8 and 246.2 ± 24.5 μM, respectively. Nitrate and NH$_4^+$ were extracted using NaCl (50 mL of 0.1 mol L^{-1} NaCl per 17.5 g of fresh sediment). Total Fe was 45.1 ± 21.3 mM, and total S was 92.9 ± 97.2 mM, both determined by digesting 200 mg dry sediment in 4 mL HNO$_3$ (65 %) and 1 mL H$_2$O$_2$ (30 %), using an Ethos D microwave (Milestone, Sorisole Lombardy, Italy). Analyses of Fe and S from digestates were carried out using an inductively coupled plasma emission spectrophotometer (ICP-MS; IRIS-OES model Intrepid II XDL; Thermo Fisher Scientific, Waltham, MA, USA). The water column (13 cm, 2 L) was continuously refreshed by pumping 3.6 L fresh medium d^{-1} from black containers into the aquaria using peristaltic pumps (Masterflex, 7015-20; Cole-Parmer, Vernon Hills, IL, U.S.A.). The water level in the aquaria was kept constant at 13 cm above the sediment using an overflow system.

"Low", "Medium" and "High" plant densities were obtained by carefully planting 1 (in the middle of the aquarium), 4 (square formation) and 16 (four rows by four columns) *L. dortmanna* plants, respectively. Plants were collected from Barstadvatn (Norway, 58°24′03″N, 6°16′01″E), and the initial dry mass of individual plants was 48.6 ± 33.0 mg. To stimulate sulfate reduction and thus the formation of sulfide, 20 g of cellulose was dissolved in 200 mL of demineralized water and carefully injected into all the sediments. Prior to the injection, the cellulose solution was bubbled with nitrogen to achieve anoxic conditions.

Plant analyses

After 3-month exposure to the three treatments, plants were counted and actual photosynthetic efficiency was measured at the middle of the 3rd, 6th and the oldest leaves of *L. dortmanna* plants by using a pulse-amplitude modulated chlorophyll fluorometer (Walz, Junior-PAM-200, Effeltrich, Germany). The mean of the three measurements was used for the analysis of the data. Actual photosynthetic efficiency was measured, in each aquarium, on one plant at low plant density treatment, and on four plants, representative for the treatment, at medium and high plant density treatments. All plants were carefully harvested and washed with demineralized water. Mortality, expressed as % of dead plants per aquarium, was measured counting the number of dead plants. A plant was considered dead when its actual photosynthetic efficiency was <0.3 and its tissues were visually substantially damaged; that is, >50 % of the plant tissue was discolored (close to white) and/or in a (partly) decomposed state (loss of internal cellular material). Live plants were then divided into shoot, root and corms, and dry mass (24 h drying at 70 °C) of each plant was measured. Plant biomass was expressed as the dry mass of live plants per m^{-2} and individual plant mass.

Sulfide intrusion is difficult to measure directly (Pedersen et al. 2004) and thus, we measured total S and δ^{34}S and used these as indicators of H$_2$S intrusion (Mascaró et al. 2009). While total S could

indicate exposure to H_2S, caution must be taken since low total S values can result from both low exposures to H_2S or high growth rates where S is diluted in fast-growing tissues (Christiansen et al. 1985). Moreover, total S does not allow determining whether S enters into the plant as SO_4^{2-} or as H_2S. Thus, we analyzed $\delta^{34}S$, which is an excellent indicator of H_2S intrusion where a low $\delta^{34}S$ signal indicates H_2S intrusion since the respiration of SO_4^{2-} by reducing bacteria results in more of the lighter sulfur isotopes (Fry et al. 1982; Frederiksen et al. 2008). To measure total sulfur (S) and isotopic sulfur composition ($\delta^{34}S$), plant material was dried and homogenized (Ball mill MM301, Haan, Germany). For total S analysis, 200 mg of dried and homogenized material was digested in 4 mL HNO_3 (65 %) and 1 mL H_2O_2 (30 %) using a mega microwave (Milestone type MLS 1200, Sorisole Lombardy, Italy). The digestives were analyzed using an ICP-MS as above. For the sulfur isotopic composition ($\delta^{34}S$) analysis, homogenized dry leaf and root material was weighed into tin capsules together with vanadium pentoxide. Sulfur isotopic composition was measured using elemental analyzer isotope ratio mass spectroscopy (EA-IRMS) at Iso-analytical (Iso-Analytical Limited, Crewe, Cheshire, UK). Due to the limited amount of plant material available, only one replica from each treatment was analyzed for sulfur isotopic composition.

Surface water and porewater analyses

Surface and sediment porewater samples were collected for chemical analysis monthly. Surface water samples were collected by submerging a bottle (50 mL) in the surface water. Porewater samples were collected without air contact with 5-cm-long sediment moisture samplers (Rhizon SMS-5 cm; Eijkelkamp Agrisearch Equipment, Wageningen, The Netherlands) that were installed vertically at a depth of 1 to 6 cm and connected to 120-mL vacuum serum bottles. Samples were analyzed for pH, alkalinity, total S, total inorganic carbon (CO_2, HCO_3^- and CO_3^{2-}) and CH_4, NO_3^-, NH_4^+ and PO_4^{3-} (Table 1; see stats in caption of Table 1). Alkalinity and pH of the water samples were measured using a Radiometer titrator (TIM 840 titration manager, Villeurbanne, France) Total S was measured using ICP-MS as described above. Total inorganic C was measured by infrared analysis (Advance Optima Infrared Gas Analyzer, ABB, Cary, NC, USA). Concentrations of NO_3^-, NH_4^+ and PO_4^{3-} were measured by colorimetry (Auto Analyzer, model III, Bran & Luebbe, Norderstedt, Germany) using hydrazine SO_4^{2-} (Kamphake et al. 1967), salicylate (Grasshoff and Johannsen 1972), ammonium molybdate (Henriksen 1965), respectively. In porewater, H_2S concentrations were measured by collecting 10 mL of porewater (extracted as previously explained), immediately fixed with 10 mL of H_2S

Table 1 Surface water and pore water characteristics as a function of surface water treatments

	Control	Alk	SO_4^{2-} + Alk	$H_{df = 2, 33}$	P
Surface water					
pH	5.9 ± 0.3[a]	7.4 ± 0.3[b]	7.3 ± 0.2[b]	23.8	***
Alkalinity (µmol L^{-1})	155 ± 42[a]	1,423 ± 966[b]	1,408 ± 1,006[b]	23.4	***
Total S (µM)	74 ± 45[a]	54 ± 73[a]	1,169 ± 1,211[b]	24.5	***
CO_2 (µM)	148 ± 385[a]	59 ± 111[a]	33 ± 45[a]	2.6	ns
Pore water					
pH	4.9 ± 0.3[a]	5.4 ± 0.6[ab]	5.5 ± 0.4[b]	11.5	**
Alkalinity (µmol L^{-1})	1,315 ± 632[a]	4,299 ± 6,431[ab]	3,654 ± 1,709[b]	11.6	**
S (µM)	108 ± 98[a]	109 ± 96[a]	781 ± 944[b]	9.1	*
H_2S (µM)	0.1 ± 0.1[ab]	0.1 ± 0.0[a]	0.3 ± 0.1[b]	16.4	**
CO_2 (µM)	612 ± 1,382[a]	1,476 ± 1,261[b]	1,348 ± 1,196[b]	15.2	***

Means ($n = 12$, ± SD) of pH, alkalinity (µ moles acid equivalents L^{-1}), total S (µM), H_2S (µM), and CO_2 (µM) at the end of the experiment are shown for "Control", "Alk" and "SO_4^{2-} + Alk" treatments. Test significance is indicated by H, and the P value (ns $P > 0.05$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). Means with the same letter do not differ significantly at 95 % CI

df, degrees of freedom

antioxidant buffer containing NaOH (to convert all H_2S and HS^- into S^{2-}), Na-EDTA and ascorbic acid (van Gemerden 1984). Hydrogen sulfide concentrations were subsequently measured using an S^{2-}-specific Ag electrode (Orion Research, Beverly, CA, USA) and a double-junction calomel reference electrode (Roelofs 1991). Data for the three plant density treatments were combined in Table 1, because plant density did not significantly affect water parameters. The concentration in surface and porewater of NO_3^-, NH_4^+ and PO_4^{3-} was not significantly affected by water treatment (data not shown). On average ($n = 36$), surface water contained 7.9 ± 1.6 μM NO_3^-, 5.9 ± 2.7 μM NH_4^+ and 2.1 ± 1.9 μM PO_4^{3-}; and porewater contained 7.8 ± 3.7 μM NO_3^-, 4.7 ± 3.4 μM NH_4^+ and 0.9 ± 0.8 μM PO_4^{3-}.

Sediment analyses

The biological O_2 demand (BOD) of all sediments at the time of harvesting was measured by incubating 5 mL of fresh sediment in 50-mL glass bottles in the dark in air saturated water solution. Smart and Barko solution (Smart and Barko 1985) adjusted to 0.1 mEq was used as water solution for all sediments independently of the treatment. Sediment samples were first pre-incubated for 12 h in 5 mL of aerated water to eliminate chemical O_2 demand. Bottles were then filled with aerated water, closed and incubated in the dark under stirred condition for a known period of time (from 2 to 4 h depending on BOD). Oxygen concentrations were measured before and after incubation using an O_2 mini-electrode (OX500 Unisense, Aarhus, Denmark). Biological O_2 demand was calculated as O_2 demand per sediment volume and unit time (nmol O_2 L^{-1} s^{-1}).

Redox potential as a proxy of the prevailing electrons acceptors in the surrounding sediment provides valuable information on (a) the oxidation status of the sediment and (b) the oxygen depletion caused by sediment mineralization. Redox potential was measured monthly during daylight, 1–3 h into the light period. Measurements were taken at 1.5 cm from the middle of the aquaria at 5 depths (1.5, 3, 6, 9 and 12 cm). Redox potential was measured using a platinum electrode and a Metrohm AgCl/KCl reference electrode (Metrohm, Herisau, Switzerland) connected to a mV/pH meter (Radiometer Nederland BV, Zoetermeer, The Netherlands). The electrical

potentials measured were converted to redox potentials relative to the standard hydrogen potential measured (E_h) by adding the reference (210 mV) and correcting for temperature and porewater pH as follows:

$$E_h = (E_{measured} + E_{reference}) + (0.2 * 293 * (pH_{measured} - 7)).$$

Statistical analyses

Data were statistically analyzed by SPSS 17.0.0 (SPSS Inc., Chicago, USA), Prism 5.01 (GraphPad Software, Inc. La Jolla, USA) and Microsoft Excel 2010 (Microsoft Office, Redmond, WA). Data were transformed by $x = \text{Ln}(x + 1)$ or $x = \text{Sqrt}(x + 0.5)$ to improve the normality when necessary. Normality was checked by Shapiro–Wilk and homogeneity by Levene's test. To assess the effect of water and plant density treatments, parametric data were analyzed using one- or two-way ANOVA followed by Bonferroni's multiple comparison test. Nonparametric data were analyzed by Kruskal–Wallis or the extension of Kruskal–Wallis test (Scheirer et al. 1976), followed by Dunn's multiple comparison test. Graphs were drawn with Prism 5.01 (GraphPad Software, Inc. La Jolla, USA). Data are presented as means $\pm$ SD. Differences are considered significant when $P < 0.05$.

Results

Mortality, biomass and actual photosynthetic efficiency

The overall aim of this study was to test if the joint action of increased water column concentration of SO_4^{2-} and alkalinity, above those typical for pristine softwater lakes with healthy populations of isoetids, adversely affected *L. dortmanna*, and whether potential impacts can be counteracted by high plant density. Essentially, the combination of SO_4^{2-} and alkalinity negatively affected *L. dortmanna* and caused mortality, lowered the areal biomass and reduced actual photosynthetic efficiency (Table 2, Fig. 1). Plant density did not significantly counteract the negative effects caused by the combination of SO_4^{2-} and alkalinity. However, actual photosynthetic efficiency was significantly positively correlated to redox

potential in the sediment, indicating a positive relationship between plant performance and sediment oxidation.

The combination of SO_4^{2-} and alkalinity had a significant effect on plant mortality especially at "Low" plant density, where 75 % of the plants died (Fig. 1a–c, Table 2). In the "Control" treatment, some plants died. In contrast to the other treatments, this mortality only occurred in one replica of one specific density treatment (Medium), indicating it was very likely caused by an exceptional factor we could not account for (Fig. 1b). The combination of SO_4^{2-} and alkalinity also significantly decreased areal biomass measured as live tissue. After 3 months of optimal growth condition in the laboratory, areal biomass at "SO_4^{2-} + Alk" did not differ from the initials (Fig. 1d–f). Plant density significantly affected on areal biomass and individual plant mass (Fig. 1g–i, Table 2). While areal biomass at "Low" and "Medium" density were significantly lower than "High", individual plant mass decreased from "Low" to "High" plant density. Similarly, "SO_4^{2-} + Alk" significantly reduced actual photosynthetic efficiency (Fig. 2j–l, Table 2), which is widely used as a stress indicator (the lower the value the higher the stress; Krause and Weis 1991). Those negative effects on areal biomass and photosynthetic efficiency tended to be alleviated by increasing plant density so that on average actual photosynthetic efficiency at "SO_4^{2-} + Alk" at "Low" density was 5-fold lower than at "Alk", but only 2.5-fold lower at "High" plant density. However, plant density did not significantly affect plant performance (Table 2). The dramatic decrease in actual photosynthetic efficiency at "SO_4^{2-} + Alk" can partly be explained by the fact that actual photosynthetic efficiency was also measured on dying plants with a high proportion of damaged tissues.

Biological oxygen demand and redox potential

At the end of the experiment, potential biological oxygen demand was high in all treatments (mean ± SD = 178.7 ± 38.6 nmol L^{-1} s^{-1}). Alkalinity by itself or in combination of SO_4^{2-} did not statistically differed from "Control" treatment (Fig. 2a–c, Table 2). Potential biological oxygen demand at "SO_4^{2-} + Alk" was highest, although the effect was significant only at "High" plant density compared to "Alk" treatment (Fig. 2a–c).

Table 2 Effects of water quality, plant density and their interaction on plant mortality, total biomass, individual plant mass and actual photosynthetic efficiency (Fv/Fm) of *Lobelia* *dortmanna* and potential biological O_2 demand (BOD) and redox potential of the sediment at the end of the experiment at several depths

	Water quality $df = 2, 27$				Plant density $df = 2, 27$				Interaction $df = 4, 27$
	F or H, P	"C"	"A"	"S + A"	F or H, P	"L"	"M"	"H"	F or H, P
Lobelia dortmanna									
Mortality	9.0*	a	a	b	1.5 ns	x	x	x	1.6 ns
Biomass	6.6*	ab	a	b	15.1***	y	y	x	2.9 ns
Individual plant mass	10.4***	ab	a	b	11.9***	x	y	z	4.1*
Fv/Fm	20.1***	a	a	b	1.0 ns	x	x	x	0.3 ns
Sediment									
BOD	5.5**	ab	b	a	1.7 ns	x	x	x	1.8 ns
Redox potential									
1.5 cm depth	6.8**	a	a	b	4.5 *	xy	y	x	1.4 ns
3 cm depth	6.7**	ab	a	b	3.9 *	x	x	x	1.2 ns
6 cm depth	14.0***	a	a	b	5.0 *	x	x	x	2.8 *
9 cm depth	11.2***	a	a	b	0.3 ns	x	x	x	1.2 ns
12 cm depth	6.9**	a	a	b	0.3 ns	x	x	x	0.9 ns

Test significance is indicated by H (for Kruskal–Wallis test) or F (for ANOVA test) and the P value (ns $P > 0.05$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). Means with the same letter do not differ significantly at 95 % CI

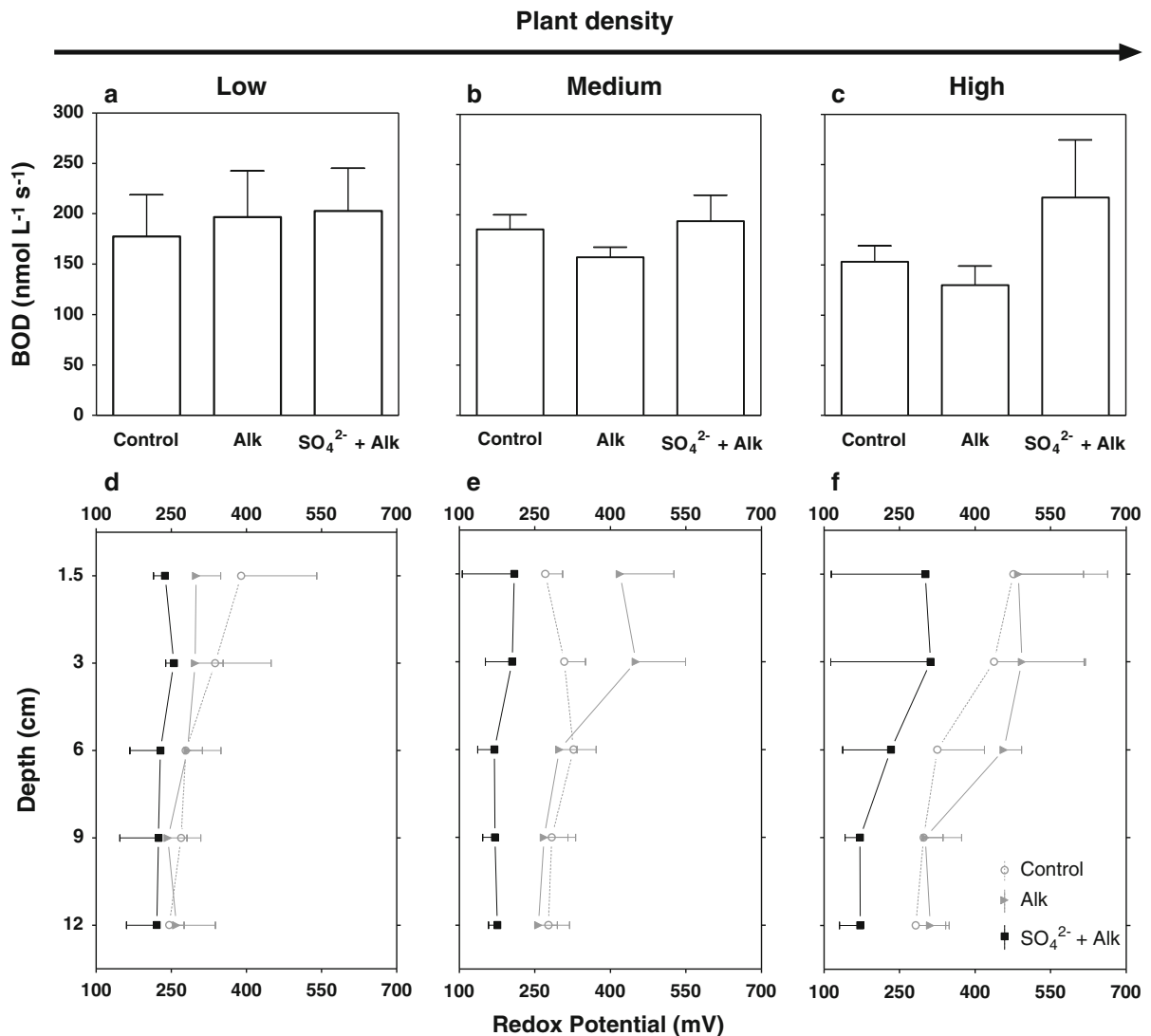df, degrees of freedom; C, "control"; A, "Alk"; S + A, "SO_4^{2-} +Alk"; L, "low"; M, "medium"; H, "high"

Plant density

Fig. 1 Plant parameters as a function of water quality at "Low", "Medium" and "High" plant density. Means ($n = 4$, SD) of mortality rate (%; **a–c**), total biomass (g DM m^{-2}; **d–f**), individual plant mass (g DM, **g–i**) and actual photosynthetic efficiency (Fv/Fm; **j–l**) of *Lobelia dortmanna* are shown for each water treatment ("Control", "Alk", "SO$_4^{2-}$ + Alk")

Sediment redox potentials at the water column treatment "SO$_4^{2-}$ + Alk" were significantly lower than at "Alk" (Fig. 2d–f, Table 2), indicating higher mineralization rates in the sediment and/or lower radial oxygen loss from plants. Plant density also significantly affected the redox potential so that sediment redox potential in the rhizosphere (up to 6 cm depth) in general increased with increasing plant density (Fig. 2d–f, Table 2). However, at "Low" density, plants were unable to oxidize the sediment and there were no visual differences in sediment redox potentials between the rhizosphere and the deeper sediment (Fig. 2d).

Actual photosynthetic efficiency was significantly positively correlated to redox potential in the sediment, indicating a positive relationship between plant

Plant density

Fig. 2 Potential biological O_2 demand (BOD, nmol L^{-1} s^{-1}) and redox potential (E_h, mV) as a function of water quality at "Low", "Medium" and "High" plant density. Means ($n = 4$, SD) of BOD (**a**–**c**) measured after harvesting, and redox potential (**d**–**f**) measured 1.5 cm from the nearest plant at several depths (1.5–12 cm) during the day time just before harvesting for "Control", "Alk", "SO_4^{2-} + Alk" treatments

performance and sediment oxidation (Fig. 3). Remarkable, 80 % of the "SO_4^{2-} + Alk" aquaria had average actual photosynthetic efficiency <0.3 threshold for dead plants in this study.

Sulfate respiration and generation of H_2S

In general, shoots and roots of *L. dortmanna* grown at "SO_4^{2-} + Alk" had a significantly higher total S content than "Control" and "Alk" treatments; only at "High" plant density, total shoot S did not differ between "SO_4^{2-} + Alk" and "Control" (Table 3). Although total S followed a similar pattern in both shoots and roots, total S in shoots was 2- to 4-fold lower than in roots. The combination of SO_4^{2-} and alkalinity resulted in $\delta^{34}S$ values lower than 5 ‰ in both shoots and roots of *L. dortmanna* (Fig. 4), although the effect was only significant in the root tissue.
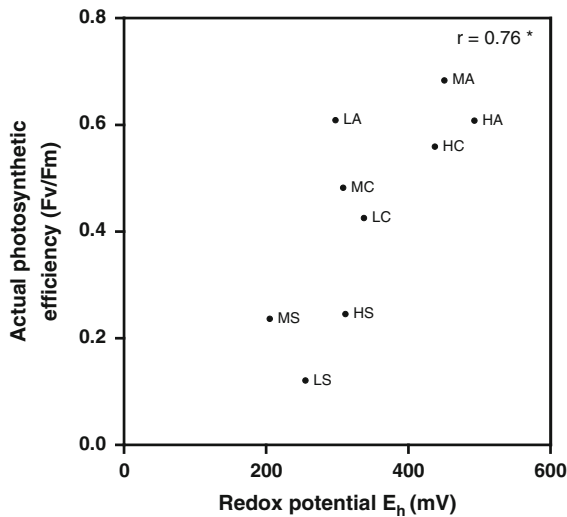
Fig. 3 Actual photosynthetic efficiency (Fv/Fm) versus redox potential. Each plot represents the mean actual photosynthetic efficiency of *Lobelia dortmanna* plants for each water ("Control" = C; "Alk" = A; "SO$_4^{2-}$ + Alk" = S) and plant density treatment ("Low" = L; "Medium" = M; "High" = H) versus the mean redox potential measured at 1.5 cm from the center of the aquarium at 3 cm of depth from the surface of the sediment. $n = 9$; r = Pearson's correlation; * $P < 0.05$

Fig. 4 Effects of water quality on δ^{34}S in shoots and roots of *Lobelia dortmanna*. Means ($n = 3$, SD) of δ^{34}S in shoots and roots are shown for different water qualities ("Control", "Alk" and "SO$_4^{2-}$ + Alk"); values represent pooled averages of "Low", "Medium" and "High" plant densities. Test significance is indicated by H, degree of freedom (*subscript*) and the P value (ns $P > 0.05$, * $P < 0.05$). Means with the same letter do not differ significantly at 95 % CI

Discussion

The combination of high SO$_4^{2-}$ and high alkalinity in surface water negatively affected *L. dortmanna* plants causing mortality, decreasing biomass and reducing actual photosynthetic. In contrast to our hypothesis, plant density did not significantly alleviate the negative effects caused by high SO$_4^{2-}$ and high alkalinity. However, there was a positive relationship between photosynthetic efficiency and redox potential in the sediment. In this section, firstly, we are discussing the
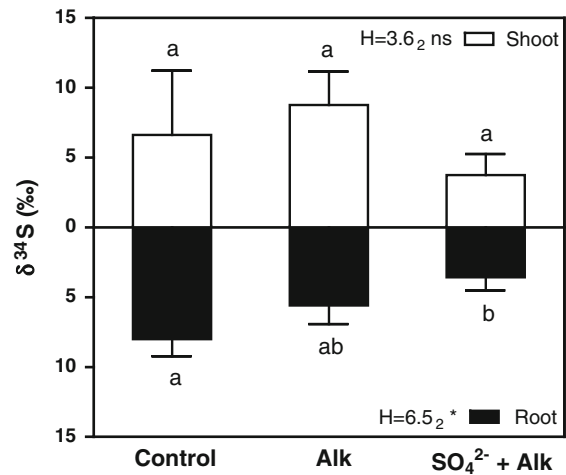
possible causes of the negative effects produced by the combination of high SO$_4^{2-}$ and high alkalinity, that is, tissue anoxia or the combination of tissue anoxia and H$_2$S intrusion in the plant. Secondly, we are addressing the lack of significant plant density effect. And finally, we are also arguing why plants were not negative affected when they were exposed only to elevated levels of alkalinity.

It is possible that long periods of tissue anoxia can explain tissue damage observed in the "SO$_4^{2-}$ + Alk" treatment (Fig. 1), especially at "Low" and "Medium" plant density. Although it has recently been

Table 3 Total S in shoots and roots of *Lobelia dortmanna* as a function of water quality and plant density

	Total S	Control	Alk	SO$_4^{2-}$ + Alk	$F_{df = 2, 9}$	P
Low	Shoot	46 < 1 [b]	22 ± 1[c]	144 ± 7[a]	97.4	***
	Root	93 ± 1[b]	91 ± 1[b]	264 ± 18[a]	351.4	***
Medium	Shoot	40 ± 2[b]	33 < 1[b]	62 ± 12[a]	16.3	***
	Root	91 ± 3[b]	98 ± 2[b]	158 ± 33[a]	14.37	**
High	Shoot	62 ± 3[a]	44 ± 10[b]	62 ± 1[a]	9.1	**
	Root	124 ± 22[a]	105 ± 9[a]	159 ± 4[b]	12.5	**

Means ($n = 4$, ± SD) of total S (µmol g^{-1} DM) are shown for the three different water qualities ("Control", "Alk" and "SO$_4^{2-}$ + Alk") at "Low", "Medium" and "High" plant density. Test significance is indicated by F, and the P value (** $P < 0.01$, *** $P < 0.001$). Means with the same letter do not differ significantly at 95 % CI

df, degrees of freedom

shown that *L. dortmanna* can tolerate short periods of tissue anoxia (Møller and Sand-Jensen 2011), longer periods of tissue hypoxia or anoxia reduce vascular translocation (Sorrell 2004), photosynthetic efficiency (Pulido and Borum 2010) and plant growth (Møller and Sand-Jensen 2011) and can even result in mortality of aquatic macrophytes (Sand-Jensen et al. 2005a; Raun et al. 2010). Leaf lacunae of *L. dortmanna* experienced anoxia for long periods in organically enriched sediments at approximately 15 °C and a plant density of c. 1,000 plants m^{-2} (Møller and Sand-Jensen 2011). In parallel, in situ experiments at similar organic addition resulted in BOD of 48 nmol $O_2 L^{-1} s^{-1}$ (Claus Møller, personal communication). Thus, it is likely that in the present study, belowground plant tissues at "SO_4^{2-} + Alk" experienced extended periods of anoxia during the night, as the plants were exposed to 4-fold higher BOD in the rhizosphere, higher temperatures and similar plant densities than in Møller and Sand-Jensen (2011) . Plants could even experience anoxia during the day at "Low" and "Medium" plant density in the "SO_4^{2-} + Alk" treatment as indicated by (a) the low redox potential (<250 mV), which is similar to the redox potential in bare anoxic sediments in softwater lakes (Wium-Andersen and Andersen 1972; Andersen and Olsen 1994) and (b) the lack of visual differences in redox potential with depth (Fig. 2, Table 3), showing that plants were unable to oxidize the rhizosphere.

The combination of anoxia and H_2S toxicity would also explain the reduced plant performance in the "SO_4^{2-} + Alk" treatment (Fig. 1). Many sediment-dwelling microorganisms will start respiring SO_4^{2-} to decompose organic matter when other energetically more favorable electrons acceptors have been depleted (Laanbroek 1990) and the SO_4^{2-} reduction results in the generation of H_2S, which is known as a strong phytotoxin (Holmer and Bondgaard 2001). Sulfide lowers photosynthesis (Pezeshki et al. 1988; Goodman et al. 1995), reduces growth (Koch and Mendelssohn 1989; Holmer and Nielsen 2007; Geurts et al. 2009), and ultimately reduces survival (Armstrong et al. 1996; Holmer and Bondgaard 2001) of wetland plants and aquatic macrophytes, even at porewater H_2S concentrations as low as 5 μM (Smolders and Roelofs 1996). In the present study, we did not detect porewater H_2S concentrations above 0.5 μM at "SO_4^{2-} + Alk" (Table 1). Nevertheless, total S as well as $\delta^{34}S$ values clearly indicate a higher H_2S

intrusion into roots in the "SO_4^{2-} + Alk" treatment in comparison with "Controls" and "Alk" (Table 3, Fig. 4). The direct comparison of $\delta^{34}S$ values with other isoetids is constrained by the lack of studies of H_2S intrusion in isoetids as the phenomenon has received little attention due to naturally low SO_4^{2-} concentrations in oligotrophic softwater lakes (Holmer et al. 1998). In addition, isoetids loose most of the O_2 produced in the photosynthesis via ROL so that the rhizosphere is often completely oxidized (Pedersen et al. 1995; Sand-Jensen et al. 2005b) and so, sulfate reduction is of little quantitative importance in isoetid populations. In seagrasses, however, H_2S intrusion has been extensively investigated and here, sulfate reduction is important due to naturally higher levels of SO_4^{2-} (Holmer et al. 2003) and much lower rates of ROL from seagrasses (Jensen et al. 2005; Sand-Jensen et al. 2005b; Møller and Sand-Jensen 2008). $\delta^{34}S$ varies among species (Frederiksen et al. 2008), but $\delta^{34}S$ values for *L. dortmanna* fall within the lower range of those observed for seagrasses (*Zostera marina*, *Posidonea oceanica*; Holmer and Nielsen 2007; Frederiksen et al. 2008, or *Cymodocea serrulata*; Povidisa et al. 2009), although they are exposed to H_2S levels more than 100-fold higher than *L. dortmanna* in the present study. Thus, we propose that H_2S intrusion in addition to tissue anoxia per se could explain the negative effects observed on plants exposed to elevated concentrations of SO_4^{2-} and alkalinity in surface water.

Plant density did not significantly counteract the negative effect produced by the combination of high sulfate and high alkalinity (Table 2). However, photosynthetic efficiency is positive correlated to sediment redox, indicating a positive relationship between plant performance and sediment oxidation (Fig. 3). On average, areal biomass and photosynthetic efficiency tended to increase from "Low" to "High" plant density at "SO_4^{2-} + Alk" treatment (Fig. 1). However, this tendency was not significant (Table 2). The increase in plant density did not result in a proportional increase in plant biomass because plant density increase caused individual plant mass decrease (Fig. 1). Therefore, the negative effects expected especially at "Low" plant density (Tessenow and Baynes 1978) were probably counteracted by the lack of plant competence, which resulted in high individual plant mass and thus high availability to oxidize the sediment.

Elevated levels of surface water alkalinity did not negatively affect plant performance (Fig. 1, Table 2). The elevated concentrations of CO_2 in the porewater of "Alk" (Table 1) could be originated from (1) enhanced mineralization processes and/or (2) from diffusion of HCO_3^- from the alkaline surface water into the sediment where HCO_3^- was then converted into CO_2 as a result of the lower pH in the porewater. Low BOD values in "Alk" treatment compared to "SO_4^{2-} + Alk" (Fig. 2) could be the result of high mineralization rates during the experiment in "Alk" treatment. High redox potential in "Alk" sediments, in comparison with "SO_4^{2-} + Alk" sediments, could promote high sediment mineralization (Ponnamperuma 1984) during the experiment, resulting in lower BOD's at the end of the experiment (Fig. 2). This hypothesis is supported by higher (although not significant) percentage of organic matter in the top layer of "Alk; Medium; and High plant densities" sediments compared to "SO_4^{2-} + Alk; Medium; and High plant densities" (data do not shown). The increase in porewater CO_2 concentrations from 0.6 mM in "Control" to 1.5 mM in "Alk" is likely to have caused the areal biomass increase as photosynthesis of isoetids does not saturate until 2–8 mM (Søndergaard and Sand-Jensen 1979; Roelofs et al. 1984; Pedersen et al. 1995). In the present study, however, competition from other plants species was excluded and in the field situation, alkalinization of the water column would likely promote the growth of fast-growing elodeids that are competitively superior to isoetids (Arts 2002; Lucassen et al. 2009; Spierenburg et al. 2009; 2010; Raun et al. 2010).

The key findings of this study have implications for the conservation and management of softwater lakes with populations of isoetids. Our results indicate that relict population of L. dortmanna may completely disappear at the time water concentrations of both SO_4^{2-} and alkalinity increase as a result of in-lake processes, seepage of polluted groundwater or runoff from the catchment. This indicates that monitoring of local groundwater and runoff is relatively important compared to monitoring of surface water quality in order to detect possible enrichment already in an early stage of the process.

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