



Diatom–salinity thresholds in experimental outdoor streams reinforce the need for stricter water quality guidelines in South Australia

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Abstract Water quality guidelines are an important tool for managing environmental pressures on freshwater streams, but guidelines are frequently set using conditions from reference sites that are assumed to be unimpacted. Using biological thresholds potentially provides a better foundation for guidelines. However, guidelines based on field observations alone may be compromised by confounding influences. This study used an outdoor stream mesocosm, an artificial substrate (rope), and six salinity concentrations to assess the veracity of a diatom–salinity threshold determined previously in natural temperate South Australian streams. In addition, shaded treatments assessed the synergistic influences of salinity and reduced sunlight. Salinity had the strongest effect on diatoms, influencing both species and functional compositions. Species diversity and richness, and functional

diversity had negative correlations with salinity. Shade strongly reduced diatom concentrations and altered species composition, with no observed interaction between salinity and light. Threshold Indicator Taxa ANalysis indicated a salinity threshold of $1610 \mu\text{S cm}^{-1}$ for both shade treatments, lower than the upper limit of the range used in current freshwater guidelines. This study recommends a new candidate guideline of $1600 \mu\text{S cm}^{-1}$ for regional freshwater streams and suggests that contemporary methods for deriving water quality guidelines may not adequately protect aquatic health.

Keywords Electrical conductivity · TITAN · Trait · Weight-of-evidence · Streams

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Introduction

Freshwater environments, including streams, provide substantial benefits for people and biota, particularly via the provision of water supply and habitats that support approximately 6% of the globe's species (Finlayson & D'Cruz, 2005; 2019; Cazzolla Gatti 2016; Abell et al., 2019). Threats to human water security and/or biodiversity are present in freshwater streams globally, including in temperate Australia (Vörösmarty et al., 2010; Dodds et al., 2013; UNESCO, 2020). Water quality is a strong determinant of freshwater ecosystem health, and land-use change can degrade freshwater ecosystems through chemical

stressors such as salinity and nutrients, affecting streams from the cellular to ecosystem level (Cañedo-Argüelles et al., 2013; Feld et al., 2018; Guariento et al., 2011; Hill et al., 2011; Millán et al., 2011), and physical pressures like altered flow, suspended sediment, and changes to light availability which is particularly important for autotrophs (Abell et al., 2019; González del Tánago et al., 2021).

Salinity, in particular, pressures osmoregulation in organisms, decreasing cellular water and nutrient uptake potential (Cañedo-Argüelles et al., 2019; Kefford, 2019). While salt toxicity is highly dependent on the organism (Kefford et al., 2004), tolerance differences between species contribute to sublethal impacts including reduced growth rates and changes to community composition, ultimately impairing biodiversity (Williams, 2001; Herbert et al., 2015). Primary salinisation occurs naturally through weathering and precipitation, groundwater contributions, and direct runoff (Cañedo-Argüelles et al., 2019). However, human activities, particularly land clearance and irrigated agriculture, can intensify this accumulation as salts stored in soils are mobilised by rising groundwaters and are brought to surface and sub-surface soils, leading to landscape and water salinisation (Williams, 2001; Cañedo-Argüelles et al., 2013; Cooper et al., 2013; Herbert et al., 2015). Secondary salinisation has been predicted to worsen in many Australian river catchments during the next centuries, a process likely amplified by climate change (Hart et al., 2003).

Monitoring stream water quality is essential for assessing ecological health and minimising anthropogenic impacts (Bunn et al., 2010; Stevenson & Pan, 2010). Managers use water quality guidelines to identify stressor concentrations that may degrade aquatic health, and determine if water is fit for purpose (e.g. for drinking, irrigation, or aquaculture). The dominant approach used in developed countries (e.g. Australia, European Union, and North America) for setting water quality guidelines is based on monitoring ambient water quality conditions from regional reference sites (Grenier et al., 2010; Birk et al., 2012; van Dam et al., 2019). Regulatory criteria are then set using statistical proportions of these data (e.g. the 80th percentile of salinity), and health is determined as a measure of the distance between a site's current condition and reference conditions. However, this approach can lack a biological basis, by

assuming reference sites are minimally disturbed and have water quality that protects their biota (Chessman, 2021).

Internationally, the reference-condition approach is often applied by defining ecological deterioration as the response of biota, rather than just changes in physical and chemical parameters (Nijboer et al., 2004; Stoddard et al., 2006; Grenier et al., 2010; Birk et al., 2012; Hess et al., 2020). Australia adopted the reference-condition approach in 2000 through a Trans-Tasman framework (ANZECC, 2000) with objectives to set regional guidelines based on local reference data where possible, with regional reference data (termed default in ANZECC, 2000) used in the absence of local information. Physical and chemical default guidelines (based on the 80th percentile) were set using monitoring data collected monthly over two years from minimally impacted regional reference sites (ANZECC, 2000; Davies, 2000; van Dam et al., 2014, 2019). By contrast, toxicant guidelines were set using biological evidence from ecotoxicology studies. Protocols for assessing biological conditions were outlined under the framework for use by monitoring authorities in addition to default guideline values when performing site assessments. However, the default physical and chemical guidelines (widely used in regular monitoring on a national scale) were mostly based on reference monitoring data.

An updated water quality framework, the Australian and New Zealand Guidelines for Fresh and Marine Water Quality, 2018 (herein: the ANZG), provides a new direction for managing aquatic habitats and recommends using physical and chemical reference data only in the absence of biological evidence to set water quality guidelines (van Dam et al., 2019). This approach uses “weight-of-evidence” to balance multiple, and varying, lines of biological evidence to set comprehensive and protective water quality guidelines (USEPA, 2016; Suter et al., 2017; van Dam et al., 2019; Mooney et al., 2020). These lines of evidence should ideally include stressor-response data from various settings, including natural streams, which are naturally variable, and experimental or laboratory evidence that reduce confounding variables (Mooney et al., 2020). In this framework, guideline values from individual studies are characterised as “candidates” to be considered in weight-of-evidence assessments (van Dam et al., 2019).

The ANZG has also revised the aquatic ecoregions where guidelines are applied. Previously, South Australia (including high rainfall temperate areas) only had generic guidelines set for arid and semi-arid waters and lacked default values for upland and temperate streams for many water quality variables including salinity (ANZECC, 2000). Ecoregions are now based on twelve major continental drainage divisions, with South Australia represented by the Murray-Darling, Lake Eyre, Western Plateau, and South Australian Gulf divisions. To this end, this study investigates if previous default salinity values which have been carried over into the ANZG framework are appropriate for the South Australian Gulf Division, and seeks to provide new biological evidence from an experimental setting to underpin water quality guidelines.

Diatoms, a class of single-celled algae, are a widely distributed organism found in almost all freshwaters. Their widespread distribution and ecological sensitivity make them ideal bioindicators of water quality. Diatoms have been widely used for stream monitoring internationally (Zhang et al., 2019) but have been underutilised in managing freshwaters in Australia (Chessman et al., 2007; Bunn et al., 2010; Soininen & Teittinen, 2019; Tibby et al., 2020). Diatom species composition reflects recent water quality conditions due to sensitivity to physical and chemical stressors (most strongly pH, salinity, and nutrients) and the relatively short lifespans of diatoms (four to six weeks) (Reid et al., 1995; Bennion et al., 2010; Julius & Theriot 2010; Stevenson & Pan, 2010; Richards et al., 2020). Diatom community function is also important for aquatic monitoring. Trait-based approaches, which utilise characteristics such as diatom growth strategy, are used to assess the ecological function (and ecological redundancy) in diatom communities (Tapolczai et al., 2016; Stenger-Kovács et al., 2018; Lengyel et al., 2020). The eco-morphological classification (B-Beres et al., 2016) combines diatom guild adaptation strategies (Passy, 2007) and morphological cell sizes (Berthon et al., 2011) to better characterise niche differentiation in analyses (Lengyel et al., 2020).

Salinity strongly influences diatom species composition (Soininen & Teittinen, 2019; Richards et al., 2020; Tibby et al., 2020; Aida Campos et al., 2021). Recent field studies in South Australian streams have demonstrated diatom–salinity

thresholds in species composition at $1000 \mu\text{S cm}^{-1}$ (Sultana et al., 2020) and $1320 \mu\text{S cm}^{-1}$ (Tibby et al., 2020). These concentrations are much lower than the upper range ($5000 \mu\text{S cm}^{-1}$) which is still used as a default value for low-rainfall, south-central Australian areas in the ANZG framework. Consequently, it has been recommended that new state-wide salinity guidelines be set at $1320 \mu\text{S cm}^{-1}$ (Tibby et al., 2020). Salinity may also negatively affect diatom cell size due osmotic pressure, impacting functional composition (Stenger-Kovács et al., 2019).

Light is a fundamental resource for autotrophs and, therefore, freshwater food webs (Lange et al., 2015; Grubisic et al., 2017), and light variability in stream reaches impacts both species composition and concentrations of diatoms (Laviale et al., 2009; Tornés & Sabater 2010; Wang et al., 2017; Liu et al., 2021). Large cell-sized autotrophs may dominate in unshaded streams (Stenger-Kovács et al., 2018), therefore light is an important confounding variable to consider when deriving salinity guidelines in regions where light availability varies markedly between streams and, or reaches.

Most recent freshwater diatom studies investigating water quality impacts have been conducted in the field (Zhang et al., 2019). However, in-field settings may experience substantial covariation between drivers (e.g. salinity and light) that hampers unambiguous assessments of water quality (Hutchins et al., 2010; ; Piggott et al., 2015; Bray et al., 2019; Kefford et al., 2022). Controlled experimental streams (e.g. mesocosms) provide uniform environments that can test specific stressors and limit confounding factors that influence the assessment of water quality variables of interest (Mooney et al., 2020; Sew & Todd, 2020; Kefford et al., 2022). Large outdoor mesocosms have this benefit but provide greater realism than laboratory experiments which are often used in ecotoxicological studies (Ledger et al., 2013; Chapman, 2018; van Dam et al., 2019).

This study aims to answer the following: (1) is $1320 \mu\text{S cm}^{-1}$ an appropriate salinity concentration for setting new regional freshwater guidelines; (2) how does shade influence the effect of salinity on diatom communities; and (3) how do diatom–salinity thresholds differ between field and experimental settings? The results contribute to a greater understanding of diatom autecology and have implications for

how freshwater guidelines are derived using biological effects data.

Methods

Study area

South Australia is the driest state in Australia (Heneker & Cresswell, 2010), and the Mount Lofty Ranges experience seasonal variability in rainfall resulting in a mix of perennial and nonperennial streams (Van Laarhoven & van der Wielen, 2009). Most permanent streams are fresh throughout the year, with electrical conductivity (EC) generally varying between 200 and 2000 $\mu\text{S cm}^{-1}$ (Anderson et al., 2019; Tibby et al., 2020). Sodium and chloride

ions dominate waters due to proximity to the coast (Blackburn & McLeod 1983; Herczeg et al., 2001; Poulsen et al., 2006). The Mount Lofty Ranges are of immense importance to the state, providing 60% of Adelaide's drinking water (Frizenschaf et al., 2015; Rashid et al., 2015) and containing a mix of residential, agricultural, and conservation land uses (Daniels & Good 2015).

To assess the effects of salinity on freshwater diatoms, an outdoor mesocosm was established at the Waite Arboretum (Adelaide, South Australia; $-34^{\circ}58'13''\text{N}$, $138^{\circ}37'49''\text{E}$) and filled with stream water ($1150 \mu\text{S cm}^{-1}$) from the Railway Dam (Belair National Park, western Mount Lofty Ranges; $-35^{\circ}0'47''\text{N}$, $138^{\circ},38',59''\text{E}$) (Fig. 1). The Railway Dam has regular inflows from Minno Creek (a second-order stream) and has similar ionic composition,

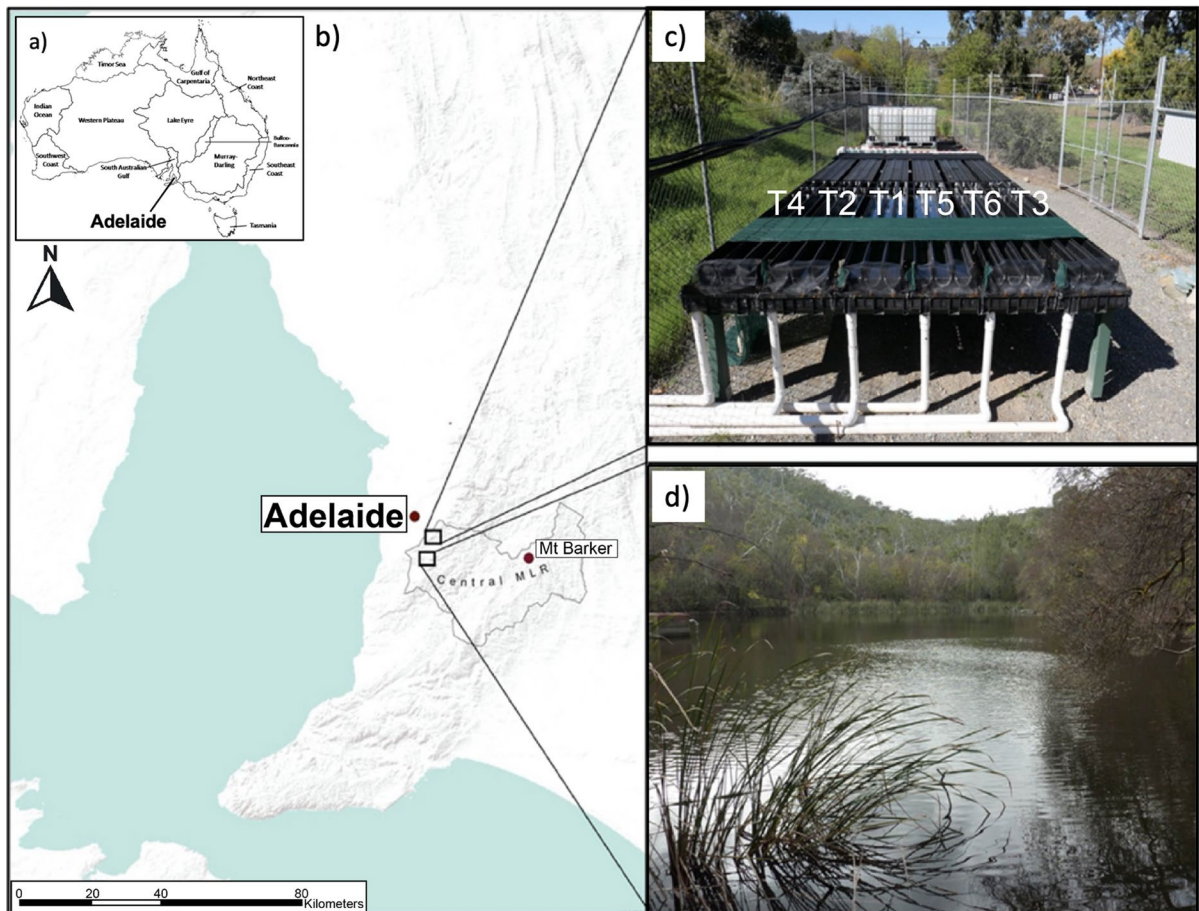


Fig. 1 a) The ecoregions where default water quality guidelines are applicable under the ANZG 2018; b) location of the study site and source water collected from Belair National Park; c) the experimental mesocosm; and d) an image of the Railway Dam

trace elements, and diatom species to streams in Belair National Park. Both sites are located in the ANZG's South Australian Gulf Division (Fig. 1).

Experimental design

The mesocosm design consisted of eighteen parallel PVC channels, with six treatments (each with three replicates) that used submersible pumps to recirculate water (see schematic in Supplementary Information), as opposed to other studies that have streamside and flow-through designs (Cañedo-Argüelles et al., 2014, 2016; Nuy et al., 2018). The experiment was run for six weeks over May, June, and July 2021, a sufficient period to allow a representative diatom community to form on either natural or artificial substrates (Kelly et al., 1998; Zidarova et al., 2020).

Salinity treatments had an even concentration distribution around the previously defined diatom–salinity threshold ($1320 \mu\text{S cm}^{-1}$). This approach increases the accuracy of threshold detection (Sultana et al., 2020). The six treatments used sodium chloride (Sunray pool salt) to achieve average conductivities of 882, 1092, 1307, 1487, 1734, and $1958 \mu\text{S cm}^{-1}$. As the source water EC ($1150 \mu\text{S cm}^{-1}$) was higher than desired in treatments 1 and 2, metropolitan tap water ($430 \mu\text{S cm}^{-1}$) was used for dilution. This water was left for 24 h in direct sunlight to release any free chlorine (confirmed with pool testing strips) before addition to the mesocosm. Water was circulated in the channels for 48 h before deploying the sampling substrates. In addition to salinity, the effects of reduced light were added as a secondary stressor with green shade cloth covering the end of each channel (shown in Fig. 1c.). The light reaching the shaded channel sections was 91% lower than the unshaded sections, a realistic representation of light penetration in forested riparian zones (Tornés & Sabater, 2010).

Frayed rope was chosen as the artificial sampling substrate in the experiment. Artificial substrates help avoid unobserved influences on diatom communities like nutrient uptake from substrates such as sediment or plants, and rope mimics epiphytic habitats for phytoplankton (Goldsmith, 1996; Elias et al., 2017; Richards et al., 2020). Rope ends were frayed to a length of 5 cm which sat below the water surface, while cable ties ensured no further fraying. Each channel had two ropes, one placed in an unshaded middle section of the channels, and one positioned

underneath green shade cloth near the end of the channels ($n=36$). The assignment of rope samples to each treatment was fully randomised. Ropes were cut above cable ties to maintain a constant sample surface area. Samples were then placed into a 50 mL centrifuge tube and taken directly to the laboratory for processing on the same day.

Water quality monitoring

EC (specific conductance; $\mu\text{S cm}^{-1}$) and temperature of each channel were measured weekly with a calibrated YSI ProQuatro multiparameter meter (YSI Incorporated, Ohio, USA). In weeks 1, 3, and 6 of the experiment, we sampled each treatment for other water quality variables, including alkalinity as calcium carbonate, bicarbonate, and carbonate, pH, turbidity, calcium, magnesium, potassium, soluble silicon dioxide, sodium, sulphate, sulphur, ammoniacal nitrogen, chloride, nitrate and nitrite as nitrogen, total nitrogen (TN), and total phosphorus (TP) (see Supplementary Information). Most of these variables were also sampled from the source water. Week 1 water quality testing occurred less than seven days after the dilution of treatments 1 and 2, so these data allow assessment of any potential water quality differences (and therefore impacts on biota) due to the addition of tap water to the two lowest conductivity treatments. Differences in luminous intensity (lux) between the unshaded and shaded treatments were quantified with four DEFI-L series meters (JFE Advantech Co., Hyogo, Japan), placed in pairs in the outside channels of the mesocosm over a ten-day period and averaged hourly.

Laboratory processing

Each centrifuge tube was filled with 35 mL of 25% hydrogen peroxide to cover the rope. Samples were placed into a sonic cleaner for 10 s to dislodge the diatoms and then in a water bath at 80°C for 4 h to remove organic matter and dislodge any remaining diatoms. Ropes were then rinsed with RO water to remove any remaining diatoms. Samples were centrifuged for four minutes at 1200 RPM and rinsed three times. Microscope slides were prepared using a micropipette and two measured aliquots of 400 μL and 800 μL mounted using the high refractive index resin Naphrax (Brunel Microscopes, Chippenham,

UK). One sample (salinity treatment 4; channel 1; shaded treatment) was damaged during the laboratory processing.

Diatom analyses

A Zeiss Axioscope light microscope (Carl Zeiss AG, Oberkochen, Germany) was used at 1000× magnification to identify 300 diatom valves per sample using Krammer & Lange-Bertalot, (1986, 1988, 1991a; 1991b) and Sonneman et al. (2000). Species counts were expressed as relative abundances to characterise species and functional composition. Diatom concentrations (cm^{-1} rope) were also calculated using the areal and volumetric proportions of samples (i.e. microscope slides, coverslips, aliquots, and sampling ropes), transect lengths (μm) needed to count 300 valves, and the microscope objective.

Diatom species were assigned to functional traits using relevant literature. Twenty eco-morphological groups (B-Beres et al., 2016), based on combining four ecological guilds (Passy, 2007; Rimet & Bouchez, 2012) and five cell sizes (Berthon et al., 2011), were used to assess diatom functionality across the treatments. The diatom guilds are low-profile, high-profile, motile, and Planktonic, while cell sizes are S1 to S5 (smallest to largest; e.g. MS2, motile size 2). See Supplementary Information for the classification of taxa.

Data analyses

All data were processed in R programming (R Core Team 2021). Two-way permutational analysis of variance (PERMANOVA) (Anderson, 2001; McArdle & Anderson, 2001) and nonmetric multidimensional scaling (NMDS; Bray–Curtis dissimilarity measure) were used to assess the species and functional composition (relative abundances) dissimilarity between diatom communities in each salinity and shade treatment. Homogeneity of multivariate dispersion (Anderson, 2006) was tested to ensure that measured effects were between groups (i.e. treatment effects) rather than due to within-group dispersion (Anderson, 2001; Anderson & Walsh, 2013). These analyses were performed using the packages “vegan” (Oksanen et al., 2020) and “ggplot2” (Wickham et al., 2016).

Species richness and diversity (Shannon-diversity index) were calculated for each sample using “vegan” (Oksanen et al., 2020). Functional diversity was determined with the functional dispersion index using the “FD” package (Laliberté et al., 2022). This distance-based index (using principal coordinate analysis and Gower’s dissimilarity) measures the average distance of each species to the abundance-weighted centroid of all species in a community in functional space (Laliberté & Legendre, 2010). Analogous to multivariate dispersion (Anderson, 2006), functional dispersion helps determine how functionally clustered a community is under given environmental conditions. Importantly, changes to species richness do not influence this index (Laliberté & Legendre, 2010).

Statistical tests were performed on all water quality and community indices. Data were first tested for normality, homogeneity of variance, and then assessed either with one-way analysis of variance (ANOVA; Welch’s or Fisher’s ANOVA) or Kruskal–Wallis test by ranks using the R package “ggstatsplot” (Patil, 2021). $P \leq 0.01$ was classified as sufficient evidence in this study. In addition to this “binary” decision-making, recently critiqued (Muff et al., 2022), we report the actual P values (rounded for brevity) and utilise the evidence descriptors recommended in Muff et al. (2022). Correlation between water quality variables, and relationships between community indices and EC, were assessed with Spearman’s rank correlation (r_s).

Threshold Indicator Taxa ANalysis (TITAN) (Baker et al., 2020) was used to assess diatom–salinity thresholds with the R package “TITAN2” (Baker et al., 2020). TITAN uses nonparametric change-point analysis, Indicator Species Analysis (Dufrêne & Legendre, 1997), and standardised z scores to integrate occurrence, abundance, and directionality of relative abundance data along an environmental gradient. TITAN measures positive ($z+$) and negative ($z-$) responses independently to quantify tolerant and sensitive species, respectively, and tracks cumulative responses (fsumz) to determine community thresholds at the 5th, 50th, and 95th percentiles (Baker & King, 2010). We determined salinity change points in separate analyses of each shade treatment and in a combined dataset using all samples.

Results

Water quality variation

EC distribution was slightly uneven with a median of $1411 \mu\text{S cm}^{-1}$, compared to the desired $1320 \mu\text{S cm}^{-1}$ (Fig. 2). There was very strong evidence that EC measurements differed between the treatment systems (Kruskal–Wallis, $H_5 = 103.37$, $P < 0.001$). There was strong evidence that both potassium (ANOVA, $F_{5,12} = 8.703$, $P = 0.001$) and alkalinity as carbonate (ANOVA, $F_{5,12} = 5.172$, $P = 0.009$) differed between the conductivity treatments (Table 1). Spearman correlation analysis provided little or no evidence that EC, TN, TP, pH and temperature were correlated (see Supplementary Information).

Diatom responses to salinity and shade treatments

Motile *Nitzschia* species were present in most samples (Fig. 3), and the nine most abundant species, namely *Nitzschia paleacea*, (Grunow) Grunow 1881; *Achnanthydium minutissimum*, (Kützing) Czarnecki 1994; *Nitzschia palea*, (Kützing) W.Smith 1856; *Ulnaria acus*, (Kützing) Aboal 2003; *Navicula veneta*, Kützing 1844; *Nitzschia*

incognita, Legler & Krasske 1941; *Nitzschia frustulum*, (Kützing) Grunow 1880; *Rhopalodia gibba*, Kützing 1844; and *Halamphora veneta*, (Kützing) Levkov 2009; respectively, were observed in both shade treatments. More diatom species were found in the unshaded channels, while the relative abundances of dominant taxa, including *N. palea*, *N. paleacea*, and *A. minutissimum*, were much higher in shaded samples. *Fragilaria crotonensis*, Kitton 1869, was most abundant in treatments 1 and 2, but was largely absent in higher conductivity treatments. Similarly, *R. gibba* and *Hantzschia amphioxys*, (Ehrenberg) Grunow 1880, were more abundant in lower salinity treatments, while *Halamphora veneta* was very rare outside of treatment 1 and treatment 3 samples. *U. acus* was abundant in several assemblages; however, relative abundance tended towards lower conductivities. *Navicula veneta* was very abundant in the two highest salinity treatments, despite being common in most other channels. *Eolimna minima*, (Grunow) Lange-Bertalot 1998, was also most abundant in treatment 5 but was rare outside these channels. *Nitzschia palea* was most dominant in treatment 6, having a stark, much higher, relative abundance in the highest conductivity streams. *N. paleacea* and *A. minutissimum*

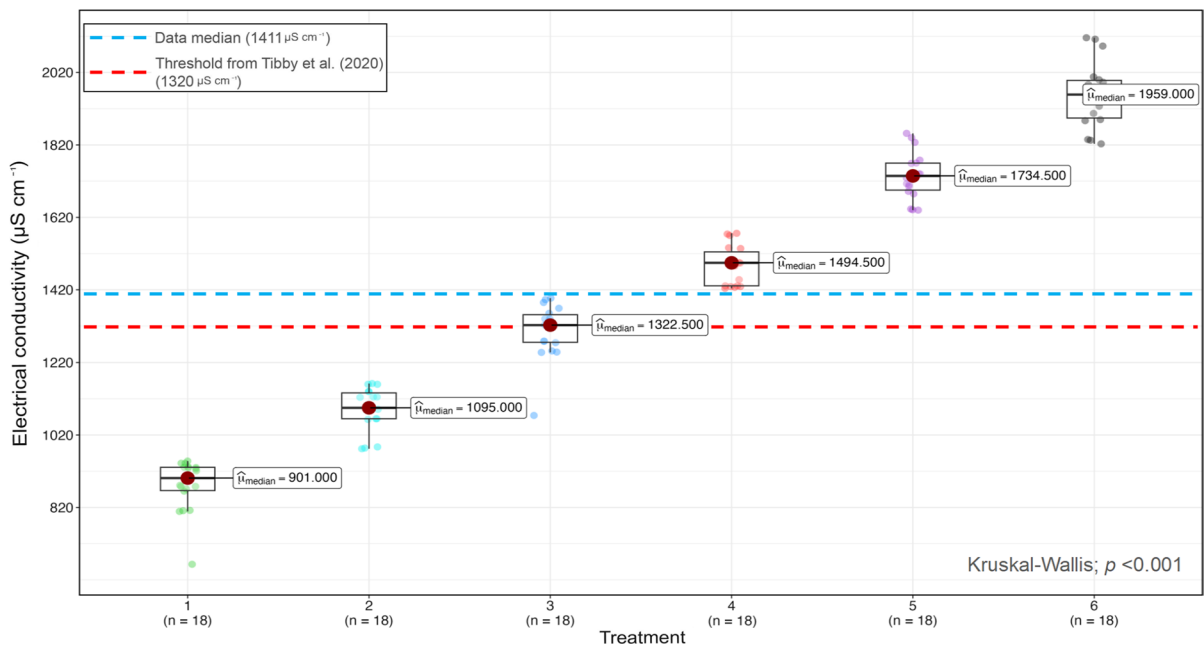


Fig. 2 Boxplot of all EC measurements taken from each channel. Data points are jittered to avoid overplotting along the x-axis

Table 1 One-way ANOVAs (Welch's or Fisher's ANOVA; df,df) and Kruskal–Wallis test by ranks (df) of water quality between the EC treatments, and a summary of water quality measurement data

	Statistical testing				Data summary		
	Test statistic	df	Effect size	P	Min	Max	Mean \pm s.d
Alkalinity as calcium carbonate (mg/L)	10.08	5	0.593	0.073	116	285	227 \pm 53
Alkalinity as bicarbonate (mg/L)	10.31	5	0.606	0.067	141	316	255 \pm 54
Alkalinity as carbonate (mg/L)	5.17	5,12	0.537	0.009	0	17	11 \pm 5
Ammonia (mg/L)	1.23	5	0.072	0.942	0.005	0.011	0.007 \pm 0.0016
Calcium (mg/L)	2.82	5,12	0.335	0.066	27	46	35 \pm 5.7
Electrical conductivity (μ S cm^{-1})	103.37	5	0.966	1.0297e-20	663	2116	1410 \pm 372
Discharge (L/min)	9.64	5	0.567	0.086	10	11.4	10.7 \pm 0.46
Magnesium (mg/L)	12.62	5	0.743	0.027	20	51	41 \pm 9.6
Nitrate + nitrite as N (mg/L)	3.4	5	0.2	0.639	<0.003	0.004	0.004 \pm 0.0005
pH	7.88	5	0.463	0.163	8.3	8.7	8.57 \pm 0.12
Potassium (mg/L)	8.70	5,12	0.681	0.001	3.4	6.5	5.4 \pm 0.85
Silicon dioxide (mg/L)	11.42	5	0.671	0.044	7.2	18.7	15 \pm 3.6
Sulphate (mg/L)	12.72	5	0.748	0.026	11	29	15.7 \pm 5.1
Sulphur (mg/L)	12.72	5	0.748	0.026	3.7	9.6	5.2 \pm 1.7
Temperature ($^{\circ}$ C)	2.37	5	0.022	0.796	12	14.5	13.3 \pm 0.8
Total nitrogen (mg/L)	5.35	5	0.315	0.375	0.35	0.93	0.58 \pm 0.15
Total phosphorus (mg/L)	4.66	5	0.274	0.458	0.009	0.048	0.020 \pm 0.009
Turbidity (NTU)	13.25	5	0.779	0.021	0.56	3.8	1.55 \pm 0.86

P values \leq 0.01 are in bold. The full water quality dataset is provided in the Supplementary Information. Effect size used a 99% confidence interval

were abundant in all samples and lacked a discernible pattern related to salinity.

Two-way PERMANOVA indicated very strong evidence for differences in diatom species composition between the conductivity treatments (Pseudo- $F_5=21.996$, $P<0.001$). There was also very strong evidence of compositional differences between shaded treatments, although these were less pronounced, compared to within-group variance, than conductivity (Pseudo- $F_1=6.502$, $P<0.001$). There was moderate evidence of an interaction between conductivity and shade, but little variance between treatments was indicated by PERMANOVA (Pseudo- $F_5=1.77$, $P=0.02$). Homogeneity of multivariate dispersion tests indicate no evidence for dispersion effects (PERMDISP, Pseudo- $F_5=0.647$, $P=0.673$) in the diatom data across the conductivity treatments, and marginally weak evidence for the shade treatments (Pseudo- $F_1=4.037$, $P=0.051$).

NMDS (Fig. 4) indicates that species from conductivity treatments 1, 5, and 6 were the most distinct from each other and the remaining treatments.

Treatment 6 had the most distinct species composition, while samples from treatment 5 were the most alike in a given treatment. NMDS clustered samples from treatments 2, 3, and 4 together; however, treatment 2 was more similar to treatment 4 rather than treatment 3. Therefore, species compositions immediately above and below the 1320 μ S cm^{-1} threshold were not notably dissimilar, but rather the greatest dissimilarity was between treatment 4 and treatment 5. NMDS indicated different positioning of shade treatments (within each conductivity-treatment cluster); however, salinity clearly had the greatest influence on diatom species composition.

Diatom concentrations between conductivity treatments in either shade category were not different, while concentrations were substantially higher in the unshaded treatment by an average factor of 2.7:1 (Kruskal–Wallis, $H_1=16.351$, $P<0.001$). Species richness was highest in treatment 1 channels and lowest in treatment 6 (ANOVA, $F_5=18.388$, $P<0.001$; Fig. 6), and there was robust evidence of a strong negative correlation between species richness

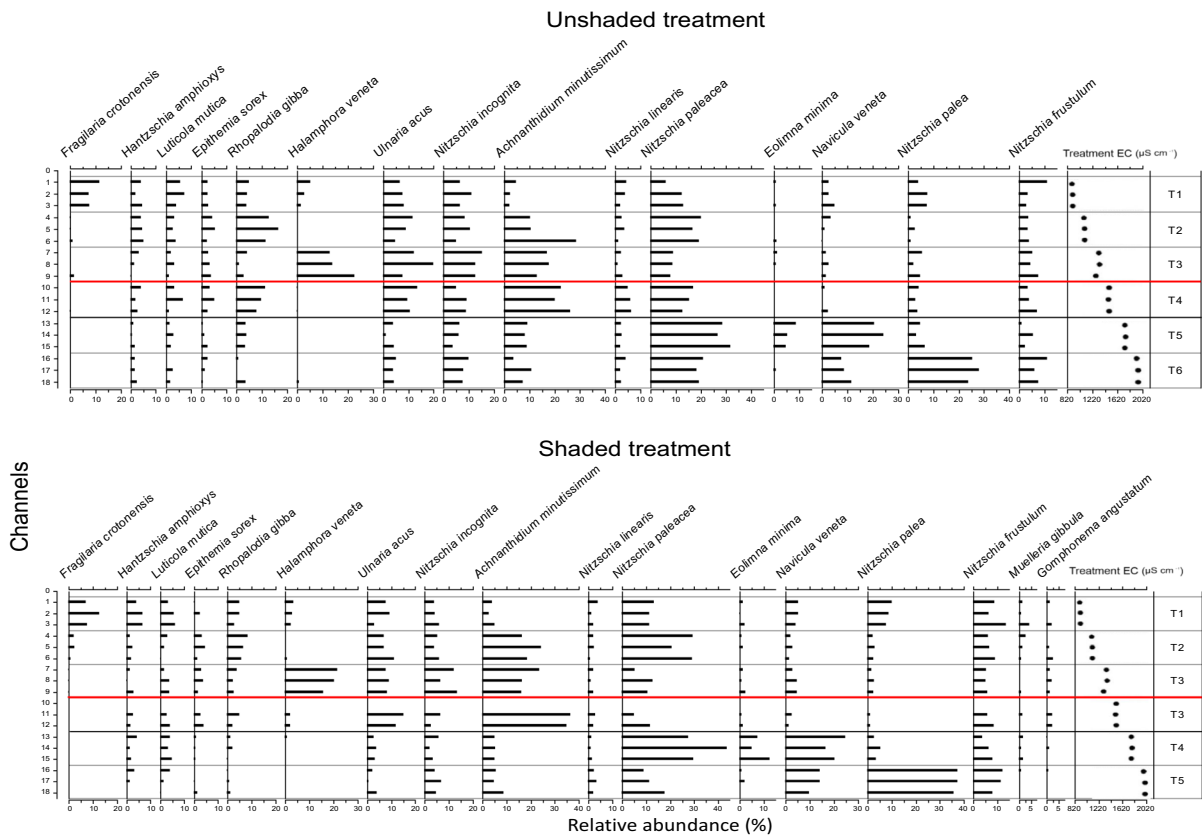


Fig. 3 Diatom assemblages in all treatments. Dashed horizontal lines represent the divisions of experiment channels and the conductivity treatments. The red dashed line represents the threshold of $1320 \mu\text{S cm}^{-1}$ from Tibby et al. (2020)

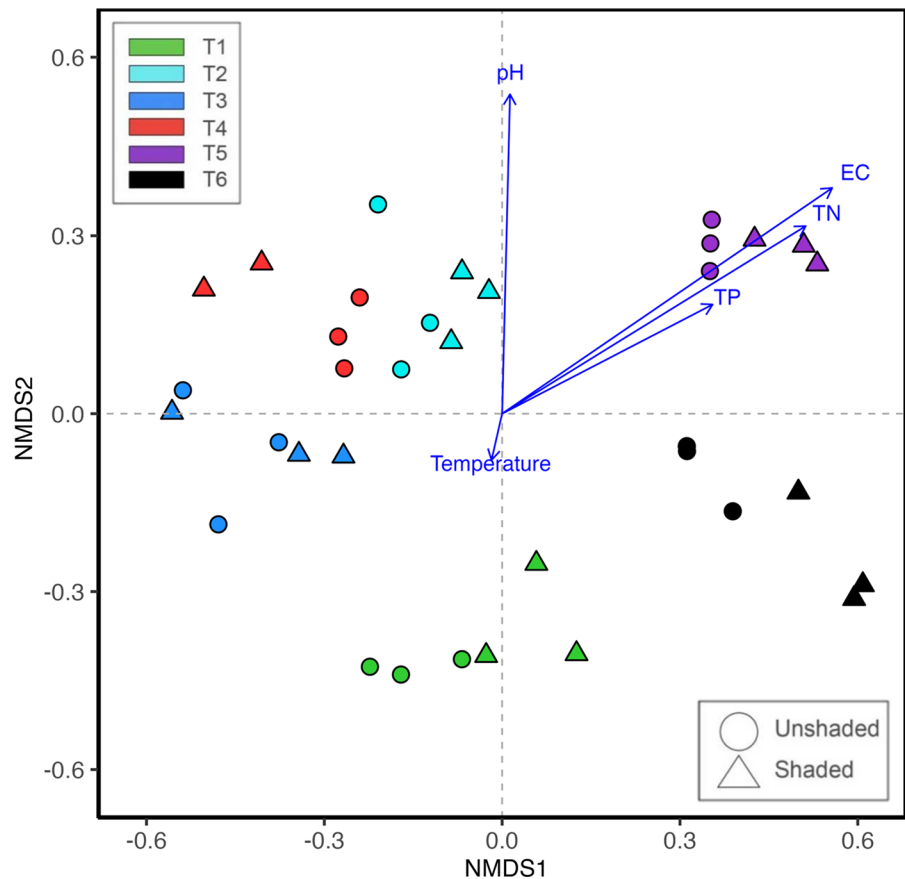
and EC (Spearman’s rank, $r_s = -0.84$, $P < 0.001$). The Shannon-diversity index was highest in treatment 1 (Kruskal–Wallis, $H_5 = 27.003$, $P < 0.001$), and there was a strong negative correlation between species diversity and EC (Spearman’s rank, $r_s = -0.87$, $P < 0.001$). There was no evidence that species richness or diversity were different between shade treatments.

Two-way PERMANOVA demonstrates that the strongest influence on trait composition was conductivity (Pseudo- $F_1 = 34.174$, $P < 0.001$). There was also strong evidence for the effect of shade on trait composition (Pseudo- $F_1 = 6.979$, $P = 0.002$), but no evidence of an interaction (Pseudo- $F_5 = 1.749$, $P = 0.069$), comparable to the results of the species PERMANOVA. There was no evidence of multivariate dispersion in the trait compositions between each conductivity and shade treatment. NMDS analysis (Fig. 5) of the functional composition demonstrates

some relationships between small motile diatoms and conductivity. Motile small diatoms (MS2) diatoms plotted closely to samples from treatment 5 and treatment 6. Relative abundances of S3, S4, and S5 diatoms from the low, high, and motile guilds tended to plot with the lowest salinity treatments.

There was strong evidence of differences in functional dispersion between the conductivity treatments (Kruskal–Wallis, $H_5 = 29.168$, $P < 0.001$), with a notable decline at, or above, treatment 4 conductivity ($1460\text{--}1500 \mu\text{S cm}^{-1}$) (Fig. 6). Treatments 5 and 6 clearly had the lowest functional dispersion, with the lower treatments having similar mean index values. Based on this measure, there was strong evidence that functional diversity had a moderate negative correlation with EC (Spearman’s rank: $r_s = -0.55$; $P < 0.001$). As with species diversity and richness, there was no evidence of functional differences between shade treatments. Functional dispersion had

Fig. 4 NMDS of species assemblages in all treatments. Stress level, indicating goodness-of-fit, was 0.13 (fair)



strong evidence of a moderate positive correlation with species richness (Spearman's rank: $r_s = 0.439$; $P = 0.008$).

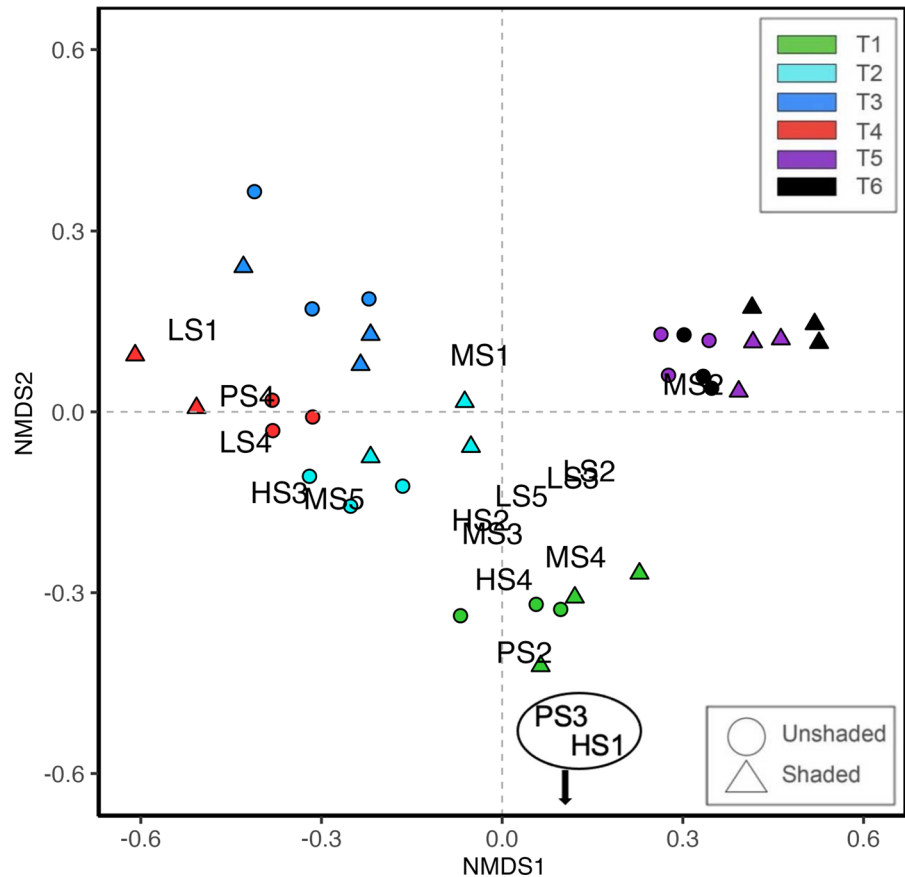
Diatom–conductivity thresholds

Table 2 shows the major change points determined by Threshold Indicator Taxa ANalysis (TITAN) in the unshaded, shaded, and combined datasets, and Fig. 7 shows the differences and range, or narrowness, of responses. In the unshaded treatment, the median of responses was $1492 \mu\text{S cm}^{-1}$ for sensitive and $1611 \mu\text{S cm}^{-1}$ for tolerant, diatom species. In contrast, the median sensitive response was higher (at $1611 \mu\text{S cm}^{-1}$) in the shaded treatment, while tolerant responses occurred lower at $1486 \mu\text{S cm}^{-1}$. Thus, the fsumz- and fsumz+ scores were alternating between the shade treatments, representing the key differences in TITAN's analysis of both datasets.

At the 5th percentile of the change point distributions, the shaded treatment had both sensitive and

tolerant responses occurring at a lower EC than in the unshaded treatment. For the 95th percentile, the differences in fsumz scores between the unshaded and shaded samples were negligible, indicating that 95% of all diatom communities had either declines in sensitive, or increases in tolerant, species occurring at, or before, $1616 \mu\text{S cm}^{-1}$. There was a narrow range of responses in both shade treatments over the EC gradient (880 to $1958 \mu\text{S cm}^{-1}$) (Fig. 7). At most, the difference between the 5th and 95th percentiles was $400 \mu\text{S cm}^{-1}$ (i.e. between the shaded fsumz- scores), indicating that change, whether in tolerant or sensitive species, occurred over a short EC range. Both the fsumz- and fsumz+ responses in the unshaded treatment were slightly left-skewed, while the fsumz- responses in the shaded treatment were highly left-skewed. Approximately 5% of sensitive diatoms responded at a lower EC than tolerant species in the shaded treatment. However, the median of sensitive change occurred higher along the EC gradient.

Fig. 5 NMDS of the ecomorphological composition from each treatment. The first letter of the abbreviations refers to the diatom guild, and the second letter and associated number refers to the size class. Guilds: M, motile; P, Planktonic; H, high-profile; L, low-profile. Size class: S1 to S5 where higher numbers represent larger diatoms. Stress, indicating goodness-of-fit, was 0.09



Discussion

Water quality variation

The value of experimental studies in freshwater ecology is that they minimise the effect of potentially confounding variables on study outcomes. There was not strong evidence of differences in water quality variables (other than conductivity) between the salinity treatments with the exception of alkalinity (as carbonate) and potassium. Statistical tests show that the differences in alkalinity as carbonate likely did not affect pH variation between conductivity treatments. Previous studies suggest that potassium concentrations in this study (3.4 to 6.5 mg/L) were unlikely to affect the diatom assemblages (Jaworski et al., 2003).

Responses of diatoms to salinity and shade

Species assemblages were distinctly different between the source water and the experimental streams.

Hence, it is likely that EC was a main driver in establishing the diatom assemblages. This indicates the appropriateness of the six-week experiment length (Kelly et al., 1998; Zidarova et al., 2020), and that diatom species composition is a reliable and valuable tool for monitoring water quality over this timeframe (Reid et al., 1995; Bennion et al., 2010; Stevenson & Pan, 2010).

The salinity treatments had the strongest effect on diatom communities. Salinity increases cellular osmotic pressure in organisms by reducing growth and nutrient uptake potential (Potapova & Donald, 2003; Cañedo-Argüelles et al., 2016, 2019; Kefford 2019). Marked differences in diatom assemblages were observed between the salinity treatments over a relatively short EC gradient (880 – 1960 $\mu\text{S cm}^{-1}$) typical for permanent streams in the Mount Lofty Ranges (Anderson et al., 2019). Although diatom compositional changes in this study were likely sub-lethal, diatom responses are important ecological processes as they nevertheless affect the productivity,

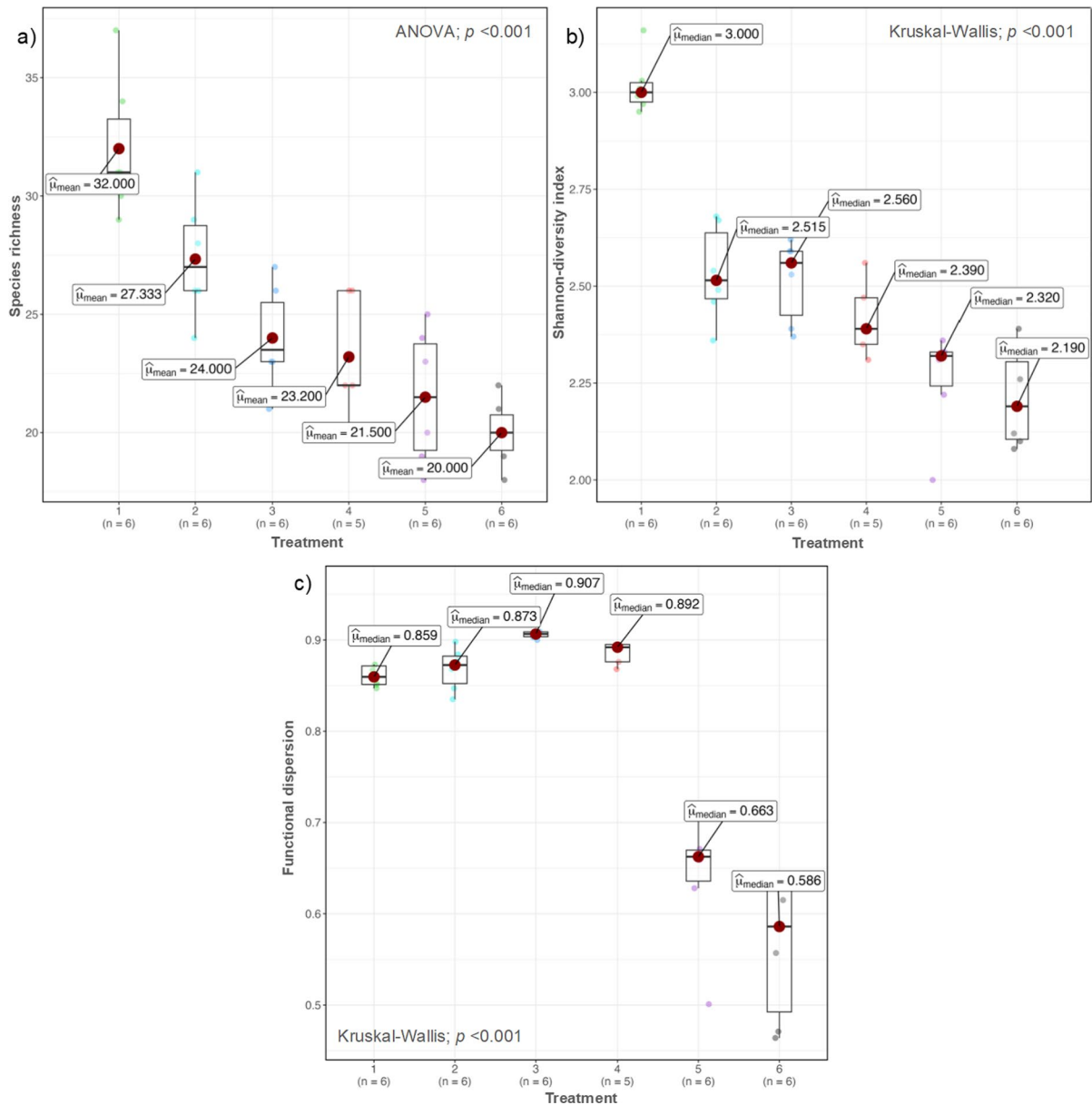


Fig. 6 Boxplots of the community metrics **a)** species richness, **b)** Shannon-diversity index, and **c)** functional dispersion observed in each treatment. Data points are jittered to avoid overplotting along the x-axis

biodiversity, and trophic interactions, leading to cascading effects in freshwater habitats (Cañedo-Argüelles et al., 2014, 2016; Herbert et al., 2015). Species richness and diversity are negatively affected by increasing salinity (Cañedo-Argüelles et al., 2019), reaffirming the need to monitor water quality in heavily modified and, or agricultural landscapes. These results are commensurate with the strong influence of

salinity on freshwater diatoms (Philibert et al., 2006; Pajunen et al., 2017; Passy et al., 2018; Soinen & Teittinen, 2019; Richards et al., 2020; Vélez-Agudelo et al., 2021).

Consideration of diatom autecology is important when using diatoms as bioindicators to manage freshwaters. In relation to increasing salinity, this experiment identified eleven negatively and

Table 2 TITAN change points along the EC gradient for diatom communities in both shade treatments, and a combined dataset

Dataset	Diatom–conductivity thresholds ($\mu\text{S cm}^{-1}$)		
	Change point (median)	5th percentile	95th percentile
Unshaded			
fsumz –	1492	1301	1616
fsumz +	1611	1406	1612
Shaded			
fsumz –	1611	1210	1616
fsumz +	1486	1324	1616
Combined			
fsumz –	1610	1091	1612
fsumz +	1611	1486	1612

three positively responding taxa. TITAN indicated a stronger response from sensitive species. *N. acicularis*, (Kützing) W.Smith 1853, and *F. crotonensis* were negatively affected between 1100 and 1500 $\mu\text{S cm}^{-1}$, consistent with their affinity for conductivities below 1500 $\mu\text{S cm}^{-1}$ (van Dam et al., 1994). *N. palea*, *N. paleacea*, and *Navicula veneta* were positive responders to salinity and are known tolerant species above 1600 $\mu\text{S cm}^{-1}$ (van Dam et al., 1994; Soininen, 2002; Karacaoğlu & Dalkıran 2017).

Rhopalodia gibba and *H. veneta* were identified as sensitive species, despite previous TITAN-based studies in South Australia suggesting these species respond positively in freshwaters between 600 and 2500 $\mu\text{S cm}^{-1}$ (Sultana et al., 2020; Tibby et al., 2020). *Nitzschia inconspicua*, Grunow 1862, is typically known as a tolerant species (Schröder et al., 2015; Tibby et al., 2020), but was sensitive in this experiment. Tolerance differences between diatom studies may result from antagonistic or synergistic effects caused by covarying chemical stressors, or biological interactions, that are inherent in natural streams (Bray et al., 2019; Piggott et al., 2015). It has been recently suggested that diatom tolerances are determined more by adaptations to local water quality rather than niche conservatism (the retention of ancestral traits) (Soininen & Teittinen, 2019), therefore, responses may vary in streams across the globe. As mesocosms can reduce confounding factors but retain certain biological interactions that occur in natural environments (Ledger et al., 2013; van Dam

et al., 2019), they would be useful for future diatom studies to test the role of niche conservatism.

Trait-based approaches are promising aquatic management tools as they assess the relative ecological contribution and redundancy of species. In addition, they enable comparison between studies with different species composition (Tapolczai et al., 2016; Stenger-Kovács et al., 2018; Lengyel et al., 2020). Functional traits may also reduce conflicting findings about the environmental preferences and, or optima of diatoms, and results between trait-based studies will remain relevant despite future taxonomic revisions (Riatio et al., 2022).

Increasing salinity in this study resulted in declines in diatom functional diversity and species richness. Functional dispersion in treatment 5 (1730 $\mu\text{S cm}^{-1}$) and treatment 6 (1960 $\mu\text{S cm}^{-1}$) was much lower than the less-saline treatments, indicating more functionally clustered diatom communities. The functional dispersion index is not affected by species richness (Laliberté & Legendre 2010), yet there was a clear positive relationship between diatom functional diversity and species richness. It has been postulated that diatom communities with more than 20 species are suitable to account for most functional traits, with greater richness resulting in ecological redundancy and a lower richness rapidly degrading functional diversities (Teittinen & Virta 2021). Our results suggest that a diatom richness above 23 (i.e. treatment 4) leads to redundancy in traits, but richness below this level (associated with conductivities above 1490 $\mu\text{S cm}^{-1}$) can result in steep declines in diatom functionality. Therefore, future experimental studies should employ longer conductivity gradients and tools such as generalised additive models to assess potential nonlinear declines and, or thresholds in the relationship between salinity, diatom functionality, and species richness.

Functional clustering at higher conductivities was evident in analyses. There was a greater propensity for more diatom traits in treatment 1 and treatment 2 (and to a certain extent treatment 4). Treatments 5 and 6 were strongly characterised by small motile diatoms (MS2), supporting the functional dispersion results. This also demonstrated some relationships between diatom cell size and conductivity as there was a greater prevalence of medium to large-sized diatoms (S3, S4, and S5) from the low, high, and motile guilds in the two lowest salinity treatments.

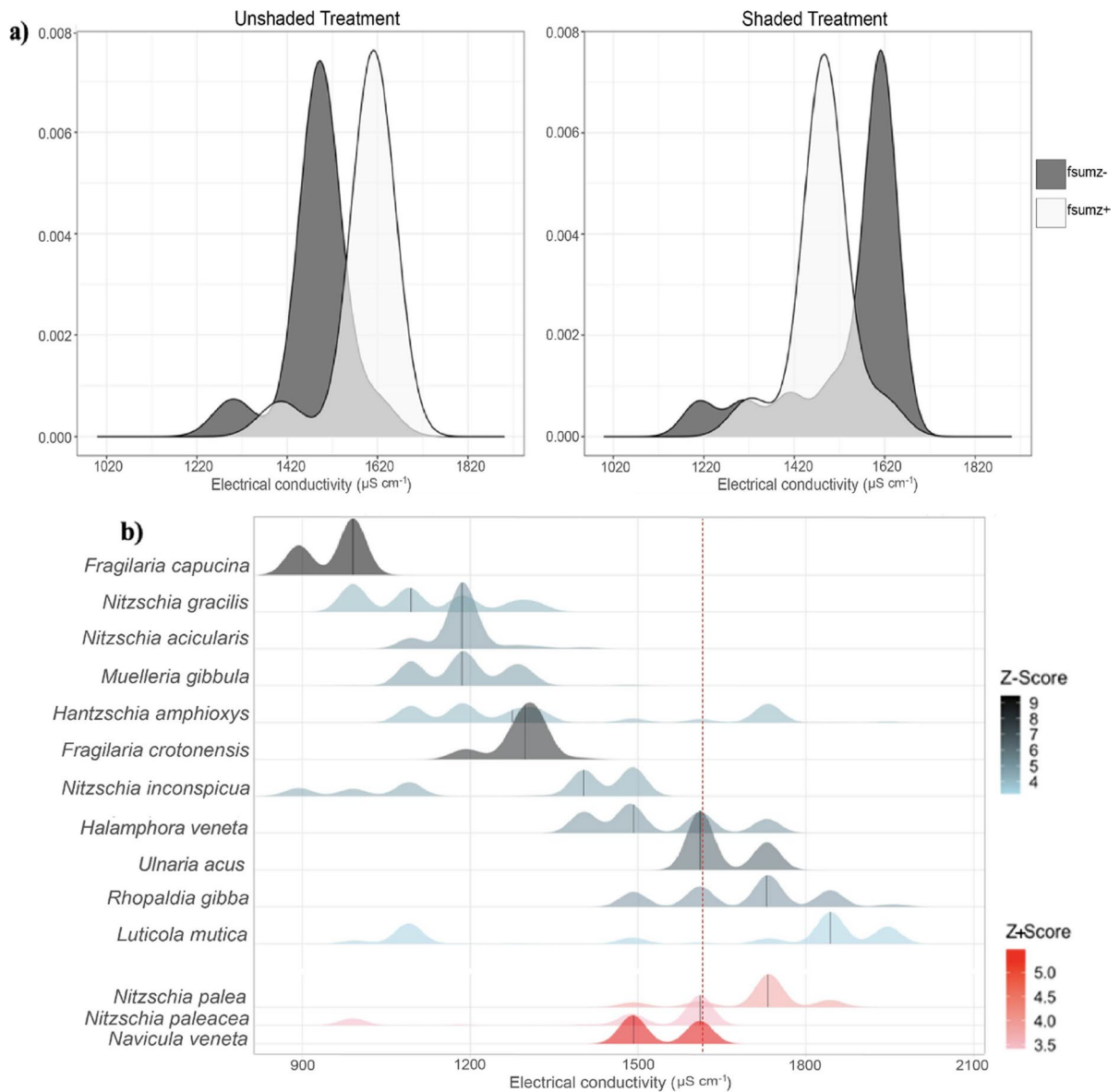


Fig. 7 a) The distribution of sensitive (fsumz-) and tolerant (fsumz+) diatom community change points from Table 2 (NB: y-axis does not reflect strength of change). b) Key taxa TITAN identified as reliable bioindicators of freshwater salinity, and

their relative abundance distribution along the EC gradient. Z-scores represent the magnitude, and emphasise the contribution, of taxon change in relation to the salinity gradient. Black lines are the species distribution median

High salinity results in cellular membrane pressure (Cañedo-Argüelles et al., 2019; Kefford, 2019) and small cell size may be a diatom adaptation response to this pressure (Stenger-Kovács et al., 2018). Stenger-Kovács et al., (2018) found that S4 diatoms are more abundant in low salinity sites, and suggested MS1 diatoms may be indicative of high salinity conditions.

However, our results were not definitive concerning the relationship between cell size and salinity, and might be due to the smaller conductivity range used in this experiment compared to Stenger-Kovács et al. (2018).

In this study, diatom community concentrations were strongly impacted by shade treatments, but

responses in species richness and diversity and functional diversity were not observed, contrasting findings from other studies (Passy, 2007; Liess et al., 2009; Stenger-Kovács et al., 2018; Lange et al., 2011). Shaded treatments (91% lower lux) reduced diatom concentrations by an average of 270%, therefore, lower light inhibited community-wide diatom growth (Tornés & Sabater 2010). Shade also strongly influenced the species composition, as observed elsewhere (Leland et al., 2001; Laviale et al., 2009; Tornés & Sabater 2010; Lange et al., 2011; Liu et al., 2021); however, there was no distinct relationship between diatom functionality and shade. Reduced-light environments are thought to favour small cell-sized and low-profile diatoms like *Achnanthydium minutissimum*, due to morphological features like higher surface-to-volume ratios (allowing greater resource-utilisation efficiency) than larger diatoms, and adaptations to lower light (Passy, 2007; Laviale et al., 2009; Hill et al., 2011; Lange et al., 2011, 2015; Leira et al., 2015).

No interaction between salinity and shade influenced either diatom taxonomic or functional composition. These were unexpected results considering that reduced light and salinity are covarying stressors for diatoms. Species diversity and richness declines were primarily restricted to salinity rather than shade treatments. Contrary to these observations, TITAN indicated that the median of sensitive diatom species began to decline at a higher EC in the shaded channels than was observed in the unshaded treatment. One reason for the TITAN result may be the increase in relative abundances of smaller size diatom species *Luticola mutica* (Mann, 1990) and *A. minutissimum* at higher EC concentrations in the shaded channels. Regardless, these results indicate the predominant influence of EC on species and functional compositions and community metrics, with shade primarily impacting diatom concentration.

Salinity thresholds in the experimental streams

This study aimed to test whether a threshold of $1320 \mu\text{S cm}^{-1}$ (Tibby et al., 2020) is appropriate as a default water quality guideline in the South Australian Gulf Division. In this experimental study, TITAN indicated that a median salinity threshold of $1610 \mu\text{S cm}^{-1}$ exists for freshwater diatoms,

regardless of shade coverage. This finding was supported by NMDS, which indicated that the two highest conductivity treatments (i.e. treatment 5, $1730 \mu\text{S cm}^{-1}$; treatment 6, $1960 \mu\text{S cm}^{-1}$) had the most distinct diatom assemblages, with the greatest difference between treatments 4 and 5 (1490 and $1730 \mu\text{S cm}^{-1}$). Hence, diatom taxonomic compositions in the artificial streams were not notably different immediately above and below the $1320 \mu\text{S cm}^{-1}$ threshold proposed by Tibby et al. (2020). The higher median threshold in this study may be due to the lack of multiple pressures on the experimental assemblages. Alternatively, an uneven environmental gradient in Tibby et al. (2020) may have led to an underestimation of the true threshold for diatoms in temperate South Australia.

Recent studies in South Australia have suggested that current default salinity guidelines for freshwater streams are set too high, and our study supports this conclusion. Sultana et al. (2019) showed that change points for stream macroinvertebrates exist at $600 \mu\text{S cm}^{-1}$, while a subsequent study in the same region found a diatom–salinity threshold of $1004 \mu\text{S cm}^{-1}$ (Sultana et al., 2020). Tibby et al. (2020) found that sensitive diatoms begin to decline at conductivities as low as $280 \mu\text{S cm}^{-1}$, while at $1320 \mu\text{S cm}^{-1}$ 95% of sensitive, and 50% of tolerant, species begin to decline and increase, respectively. While our results suggest that 50% of both sensitive and tolerant species changed significantly at $1610 \mu\text{S cm}^{-1}$, these regional studies are all consistent, demonstrating that the upper limit of $5000 \mu\text{S cm}^{-1}$, still used as the default guideline value for the South Australian Gulf Division in the ANZG, is set too high.

These findings serve as multiple and varying lines of evidence (from both field and experimental settings) for a framework (ANZG 2018) that is reliant on a weight-of-evidence approach rather than just reference conditions. Here, a candidate salinity value of $1600 \mu\text{S cm}^{-1}$ is recommended for the South Australian Gulf Division to conserve freshwater diatom communities. However, the actual value should be derived by assessing relevant evidence (Sultana et al., 2019, 2020; Tibby et al., 2020) based on wider aquatic management objectives (Suter et al., 2017; van Dam et al., 2019; Mooney et al., 2020).

Study limitations

Water quality variables other than salinity may have influenced some diatom species in this study. Treatment 5 had higher TP in week 3 and higher TN in weeks 3 and 6 compared to the other treatments. Treatment 6 had elevated TN in weeks 3 and 6. These treatments had notable relative abundances of *Navicula veneta* and *E. minima*, which are known indicators of nutrient enrichment (Cochemo et al., 2015; Delgado & Pardo, 2015; Nunes et al., 2019). Moreover, *Nitzschia palea* is widely known as a tolerant species to conductivity and nutrients (Soininen, 2002; Karacaoğlu & Dalkıran, 2017; Pajunen et al., 2017; Nunes et al., 2019) and was abundant in treatment 6 assemblages. Hence, it is difficult to determine whether the elevated relative abundances of these species are related to salinity alone.

Although sodium and chloride are the dominant ions in freshwaters in the Mount Lofty Ranges (Blackburn & McLeod, 1983; Herczeg et al., 2001; Poulsen et al., 2006), different ions dominate in other parts of the world, hampering direct comparison of diatom–salinity responses between locations (Cañedo-Argüelles et al., 2019). For example, calcium and magnesium are predominant ions in boreal and temperate streams of Europe and North America (Potapova & Donald, 2003; Soininen et al., 2004). Hence, future salinity–mesocosm studies could investigate if different ionic compositions alter conductivity tolerances, which, in turn, can help further assess the nature of diatom niche conservatism (Soininen et al., 2019; Soininen & Teittinen, 2019).

The species pool of the experiment was likely limited by the use of a single inoculum from the source water. Thresholds may change when a greater range of species (both sensitive and tolerant) are included. Future experiments should use more diverse diatom inocula by adding small amounts of a variety of substrates (e.g. vegetation and sediment) or cells concentrated in water samples from regional freshwaters.

Conclusion

Salinity in this experimental study was associated with significant changes in species composition of diatom assemblages at concentrations lower than the regional default water quality guideline value. The

greatest dissimilarity between diatom communities existed between 1490 $\mu\text{S cm}^{-1}$ and 1730 $\mu\text{S cm}^{-1}$, and Threshold Indicator Taxa ANalysis (TITAN) determined a diatom threshold at 1610 $\mu\text{S cm}^{-1}$. Moreover, functional diatom diversity had notable declines at conductivities above 1490 $\mu\text{S cm}^{-1}$. As such, a candidate guideline value of 1600 $\mu\text{S cm}^{-1}$ (with an uncertainty range of 1500–1750 $\mu\text{S cm}^{-1}$) is recommended for updated freshwater salinity guidelines applicable to the South Australian Gulf Division. Our threshold value was higher than that proposed by Tibby et al. (2020) likely due to a reduced number of pressures on biota. This demonstrates both the value of mesocosms, precisely testing biotic responses to specific water quality stressors, and their limitation, lacking realistic interactions common in field studies. Therefore, mesocosms should act only as one line of evidence in a weight-of-evidence framework such as the ANZG. Wider experiment gradients with lower conductivities (especially $< 500 \mu\text{S cm}^{-1}$) would provide more comprehensive experimental evidence for freshwater guidelines. These findings, alongside other regional field-based studies, demonstrate that water quality guideline values derived using reference-condition approaches are potentially hampering ecological management in freshwaters.

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Data availability All data are available at this online repository: <https://doi.org/10.6084/m9.figshare.19970708>

Declarations

Conflict of interest The authors have no competing interests or conflicts.

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