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Evaluating the impact of residual low chlorine concentration on phytoplankton communities by flow cytometry

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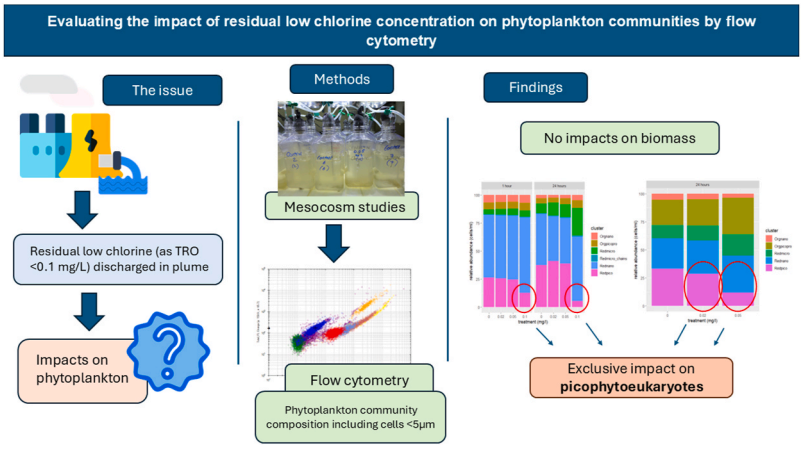
HIGHLIGHTS

- Chlorine at doses up to a maximum of 0.1 mg/L causes limited impacts on marine phytoplankton.
- Flow cytometry is an effective tool to determine impacts of chemicals on natural phytoplankton communities.
- Picophytoeukaryotes were the most sensitive group.

GRAPHICAL ABSTRACT



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ABSTRACT

Chlorination is widely used to prevent biological fouling in power station cooling water systems. It may impact non-target organisms both within the cooling system and after discharge (primary and secondary entrainment). However, there is a lack of data on the impacts of the low chlorine concentrations that occur in the discharged plume on marine phytoplankton community structure and function.

We examine the impacts on natural phytoplankton communities of single and multiple exposures to chlorination at concentrations between 0.02 and 0.1 mg/L total residual oxidants (TRO). Low-level chlorination causes limited changes in diversity and has no impact on total biomass. However, changes in size structure and functional diversity quantified using flow cytometry do show a reduction in smaller cells, particularly eukaryote picophytoplankton.

These impacts are not detectable using chlorophyll *a* concentration alone, so flow cytometry provides important additional information over more standard ecotoxicological methods.

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The effects are likely to be localised in the vicinity of the discharges (mixing zone) where the environmental quality standard (EQS) of 10 μg/L for chlorine is exceeded, but impacts on coastal food webs and biogeochemical cycles should be further evaluated.

1. Introduction

Coastal power stations dissipate excess thermal energy as heat with direct cooling through cooling water systems, requiring the intake and subsequent discharge of large volumes of water from and to the surrounding environment (Turnpenny et al., 2010). A side effect of the use of natural waters as coolant is the entrainment of organisms that can cause biological fouling and consequent obstruction of the cooling systems. A commonly used antifouling strategy is low-level chlorination (Taylor, 2006), usually in the form of adding sodium hypochlorite solution to reduce the hazards associated with chlorine gas (Atkinson, 2015). The discharged water plume is characterised by increased temperature compared to the surrounding waters and the presence of residual antifouling compounds (Dyer et al., 2017) .

In marine and estuarine environments, chlorine, applied either as gas or sodium hypochlorite, quickly generates hypochlorous acid, hypochlorite ions and chlorinated amines because of hydrolysis and reaction with ammonia. It also reacts with bromide in seawater to form hypobromous acid, hypobromite ions and brominated amines. Any chlorine and other chlorinated and brominated oxidising species remaining after the chlorine demand of seawater is met are termed Total Residual Oxidants (TRO) (Khalanski and Jenner, 2012). In freshwater, biocidal activity can be linked to the sum of free available chlorine and combined available chlorine which is termed Total Residual Chlorine (TRC). In the marine environment, due to reaction with bromide, most of the residual biocidal activity is bromine based (Atkinson, 2015). Thus, we will use TRO in this study as this is the most appropriate for saltwater.

To effectively prevent biofouling in industrial plants using freshwater or seawater, the chlorine demand must be accurately assessed to ensure that chlorination maintains sufficient levels of residual oxidants. This ensures the treatment remains effective over time. At the same time chlorine dosing levels must also minimise residual chlorine in the environment. The plume created by cooling water discharges in a tidal coastal area can affect a wider area than in a non-tidal region as the heated water moves with tidal currents. Nevertheless, at each given time, only a smaller area is impacted (Dyer et al., 2017). Non-target organisms are expected to be entrained in the plume and exposed to different conditions depending on the area of the plume they enter. This process is referred to as 'secondary entrainment' to avoid confusion with the primary entrainment of biota in the cooling water systems (Dyer et al., 2017).

Chemical discharges to the environment from various anthropogenic processes are managed by reference to quality standards based on toxicity data often produced in laboratory studies. For a coastal power station such as that proposed to be built on the east coast of England (Sizewell, UK), chlorinated discharges are expected to exceed the UK Environmental Quality Standards (EQS) for TRO, which is 10 μg/L, in an area of 338 ha (EDF Energy, 2020). This area is the 'mixing zone' in the proximity of the discharges, where exceedance of EQS is allowable (European Commission, 2010). EQSs are normally based on Predicted No Effect Concentration (PNEC) data, but there are few good quality toxicity data for marine algae from which to derive this value so freshwater study data are more heavily relied on. Methods to determine TRO concentrations in the environment have low sensitivity so the current UK EQS of 10 μg/L has been set based on the lowest concentrations that can be reliably measured in the environment (Sorokin et al., 2007). These limitations in available toxicity test data and analytical methods may therefore make the marine EQS less protective.

Phytoplankton are at the bottom of marine food webs and, together with other unicellular microorganisms, play a key role in marine biogeochemistry and ecosystem functioning (Not et al., 2012). Coastal phytoplankton communities in the North Sea are often dominated by larger cells (>20 μm) during the spring bloom (Wiltshire et al., 2008). The size structure and functional diversity of phytoplankton community are key in determining energy fluxes in marine ecosystems as well as influencing biogeochemical cycles (Le Quere et al., 2005; Schmidt et al., 2020) with higher taxonomic and functional group diversity in the smallest size-fraction (nano and picoplankton) (Ramond et al., 2019).

Different methods can be used for monitoring phytoplankton communities and the impacts of toxicants on them. Traditional approaches include measuring chlorophyll *a* concentration as a proxy for biomass (Jeffrey and Mantoura, 2005) and microscopic examination for phytoplankton diversity (Edler and Elbrächter, 2010; Utermöhl, 1958). Chlorophyll *a* data are usually combined with microscopy and other indices for an assessment of the ecological status of coastal and transitional waters (Devlin et al., 2007). High-performance liquid chromatography (HPLC) can be used for biomass estimation using chlorophyll *a*, and to determine community diversity using accessory pigments (Roy et al., 2011). These techniques, particularly those related to HPLC, are destructive, require large sample volumes and are relatively expensive and time-consuming, limiting the number of time points at which measurements can be made during an experiment. More recent approaches using flow cytometry can be used for both the determination of biomass using red fluorescence measurements and of community structure using a combination of size information and fluorescence (Haraguchi et al., 2017). The presence of photosynthetic pigments in phytoplankton cells allows autotrophic cells to be distinguished from debris and sediment (Veldhuis and Kraay, 2000). Thousands of cells can be processed in a few minutes from small volumes (Marie et al., 2005). It provides data on plankton size classes which can be used to define plankton functional groups (Salmaso et al., 2015) and allows the quantification of small cells with sizes <2 μm (Marie et al., 2005;

Table 1
TRO concentrations ± standard deviation (mg/L) measured during the single dose experiment with a natural community of phytoplankton sampled in April 2016 (single dose experiment, n = 4) and May 2016 (multiple dose experiment, n = 4). Doses 2 and 3 are only applicable to the multiple doses experiment.

Experiment	TRO (mg/L)	Dose 1		Dose 2			Dose 3	
		1 min	30 min	1 min	30 min	3 h	1 min	30 min
Single dose	0	<0.02	NA	NA	NA	NA	NA	NA
	0.1	0.04 ± 0.01	0.02 ± 0.01	NA	NA	NA	NA	NA
	0.05	0.02 ± 0.01	0.03 ± 0.01	NA	NA	NA	NA	NA
	0.02	0.02 ± 0.01	0.02 ± 0.01	NA	NA	NA	NA	NA
Multiple doses	0	<0.02	NA	NA	NA	NA	NA	NA
	0.05	0.03 ± 0.01	0.00 ± 0.01	0.03 ± 0.01	0.01 ± 0.01	0.00 ± 0.00	0.05 ± 0.01	0.02 ± 0.01
	0.02	0.00 ± 0.01	0.00 ± 0.01	0.02 ± 0.01	0.00 ± 0.00	0.01 ± 0.00	0.02 ± 0.01	0.00 ± 0.01

Thyssen et al., 2015). Flow cytometers with multiple fluorescence excitation and emission wavelengths allow phytoplankton groups to be distinguished in more detail. A widely used categorisation based on fluorescence places cells into three main groups: orange fluorescing cells (fluorescence due to the presence of phycoerythrin), red fluorescing cells and a combination of the two (red and orange fluorescence) which combined with size information can identify different phytoplankton functional groups (Marie et al., 2005). A standardised vocabulary has been recently developed for flow cytometry data to allow better comparison of data obtained by different researchers and geographical areas (Thyssen et al., 2022). Flow cytometry has been identified as a potentially valuable tool in ecotoxicology, with applications with various cell types (Wlodkowic et al., 2022), single species microalgae (Prado et al., 2009) but also phytoplankton communities (Pomati et al., 2017).

Several papers have assessed the impact of chlorination on plankton communities, but often use relatively high concentrations of chlorine (Coughlan and Davis, 1981; Davis et al., 1981). More recently, impact of primary entrainment on a community of marine phytoplankton was assessed, based on chlorination at a level of 0.2 mg/L TRO and an increase in temperature (Vannoni et al., 2021). A 90% reduction of biomass (as chlorophyll *a*) and a decrease in picophytoplankton was observed. Other data on phytoplankton are available but focused on effects on primary productivity and total chlorophyll *a* (Casas-Monroy et al., 2019; Poornima et al., 2005, 2006). More recently, the impact of ultralow-doses of chlorination (as low as 0.02 μg/L free chlorine) was investigated in the freshwater microalgae showing inhibitory effects at 0.1 μg/L NaClO for the cyanobacteria *Microcystis aeruginosa* and 0.2 μg/L for the Chlorophyceae *Chlorella vulgaris* (Cao et al., 2021). Nevertheless, there is a lack of data on natural marine phytoplankton communities exposed to the low concentrations of chlorine expected in the discharge plume.

To fill these gaps, this study examines several effects on natural marine phytoplankton communities, including changes in the size distribution of cells using flow cytometry. This enables us to evaluate likely impacts of secondary entrainment in the chemical plume associated to the cooling water discharge of a coastal power station, using chlorine concentrations expected in this context. It is hypothesised that low chlorine doses affect smaller components of the phytoplankton community, and this effect is intensified when organisms undergo multiple exposures. To test this hypothesis, two experiments were carried out using natural phytoplankton communities:

- the toxicity of a single chlorination dose of up to 0.10 mg/L TRO was tested on a spring community of phytoplankton sampled in April. This experiment aimed to evaluate the impact of secondary entrainment in the immediate vicinity of the cooling water outlet.
- the toxicity of three repetitive chlorination doses up to 0.05 mg/L TRO was tested on a spring community of phytoplankton sampled in May. This experiment aimed to evaluate the impact of multiple secondary entrainments in the outer edges of a cooling water discharge plume during a tidal cycle.

Test doses were selected based on expected concentrations in the discharge plume, specifically in the mixing zone, as estimated for a power station in the East of England (EDF Energy, 2020).

Flow cytometry was used as the dominant method to assess changes in total biomass and functional diversity in the phytoplankton communities. Data were then complemented by HPLC analysis to provide data on total chlorophyll *a* and accessory pigments for biomass and diversity measurements.

2. Methods

2.1. Test chemicals

A laboratory grade solution of sodium hypochlorite (NaOCl) containing 5% chlorine was obtained from Acros Organics (Geel, Belgium). A stock solution containing 0.05% sodium hypochlorite was prepared in reverse osmosis water and used to achieve the spiking concentrations. Chlorine in seawater is highly reactive and forms chlorine produced oxidants that are responsible for the biocidal effect in seawater. These were measured as total residual oxidants (TRO) following the colorimetric DPD (N,N-diethyl-*p*-phenylenediamine) method as recommended by APHA (1992). A Camlab colorimeter (model CW1000, Cambridge, UK) was used after calibration using chlorine reference standards purchased from Lovibond (Amesbury, UK). A zero reading for absorbance was obtained using a subsample of the test media. The detection limit for the method is 0.02 mg/L.10 ml samples of seawater were taken from each test vessel with a mechanical pipette and added to a test tube containing Hach® DPD total chlorine reagent (Hach, Salford, UK). After a reaction time of at least 3 min, the samples were filtered through a syringe fitted with a 0.2 μm filter into a 10 ml reading cell and analysed with the colorimeter. Results are reported here as TRO (mg/L).

2.2. Single dose chlorination experiment

Water samples were collected offshore of Sizewell, Suffolk, UK (52.218976 N, 1.667965 W) on April 16, 2016. 80 L of surface seawater were collected using 15 L containers; brought back to the laboratory and pooled together in a 90 L tank. The water was continuously mixed using a recirculating aquarium pump at 15 °C overnight. 2 L of the seawater were added to 20 polypropylene bottles. Sodium hypochlorite was added to achieve a TRO concentration of 0.1, 0.05 or 0.02 mg/L, with four replicates in each treatment group. TRO was measured immediately after the addition and 30 min after this. Control treatments (eight replicates) were kept at 15 °C and were not subjected to chlorination. All the replicate groups were sampled within 1 h and after 24-h of the treatment for pigment analysis, flow-cytometry measurement and PAM fluorescence. PAM fluorescence methods are in supplementary materials only.

2.3. Multiple chlorination experiment

Water samples were collected offshore of Sizewell, Suffolk, UK on the May 15, 2016 as described above. Treatment groups (four replicates each) were subjected to three sodium hypochlorite doses to achieve a TRO concentration of 0.05 and 0.02 mg/L with 3 h' interval between each. TRO was measured immediately after each addition and 30 min later. Control treatments (eight replicates) were kept at 15 °C and were not subjected to chlorination. All the replicates were sampled after 24 h of the treatment for pigment analysis, flow-cytometry and PAM fluorescence. PAM fluorescence methods are in supplementary materials only.

2.4. Test conditions

The studies were undertaken at 15 ± 2 °C in a controlled temperature room with a light-dark cycle of 16:8 h and light intensity of 60 ± 10 μmol/m^2/s. The position of the test vessels was randomised to avoid differences due to any variation in light intensity. Gentle aeration was used to keep the water mixed and avoid the settlement of the cells. Minimum and maximum room temperature was recorded daily and always fell within acceptable limits. Salinity, pH and dissolved oxygen were recorded at the beginning and at the end of each experiment.

2.5. Flow cytometry

A Cytosense flow cytometer (Cytobuoy, Woerden, The Netherlands) equipped with a 488 nm argon laser using USB5.5.1.8 © software was used to determine the concentration of phytoplankton cells in the test replicates. Samples were analysed during 5 min at 4.7 μl/s by triggering

on the red fluorescence (TL: 18) which allowed distinction of phytoplankton functional types. 1 μm and 3 μm standard beads were added to each sample. Flow cytometer data were presented in two-dimensional cytograms using different parameters such as forward and sideward light scatter, red fluorescence and orange fluorescence to define clusters as described in Thyssen et al. (2022). In this study, we analysed the results using Cytoclus 3.7.15.4© software to determine the number of cells per ml, the size calculated from the mean of the length forward scatter (FWS) for each cell. The forward scatter size was transformed as units of carbon following the equation established in Owen (2014) and used in (Vannoni et al., 2021) and shown in Supplementary material.

2.6. Statistical analysis

Statistical analysis on size distribution, cells/ml and estimated carbon were performed using R version 4.02.2 with vegan (version 2.5–7) and rstatix (version 0.7.0) packages. Size distribution data were transformed using a $\log_2$ transformation prior to statistical analysis. The transformed data were then divided in size classes of 0.25 (to create 4 bins for every doubling of cell size) and analysed using analysis of similarities (ANOSIM) (Barry et al., 2021; Clarke, 1993). Resemblance was calculated using Euclidean distance. Cells/ml, estimated carbon and chlorophyll variable fluorescence data (included in supplementary materials) were visually inspected to determine if data met normality and homogeneity of variances assumptions which were not met. Data were analysed using non-parametric Kruskall-Wallis test (Hollander and Wolfe, 1973) followed by Dunn's multiple comparison test (Dunn, 1964). Detailed statistical results are included in the supplementary materials. Changes in phytoplankton functional groups composition and accessory pigments relative to chlorophyll *a* were analysed using the PRIMER package v7 (Clarke and Gorley, 2015). Similarity between samples was explored through hierarchical clustering analysis as well as non-metric multi-dimensional scaling (nMDS) based on rank similarity matrices using Bray–Curtis coefficient. Statistical differences were calculated using ANOSIM. No outliers were identified or removed for statistical analysis.

3. Results

Chlorine demand and decay was measured in preliminary studies to help inform chlorine dosing for the main study. Additional data on chlorine demand and decay run in parallel to the two experiments is included in supplementary materials for a spike of 0.2 mg/L TRO (Table S4 and Table S5). Due to limit of detection of the instrument the dosing for the chlorine demand used a 0.2 mg/L spike. Chlorine demand was higher for the seawater used for the single dose experiment in which TRO decayed from 0.2 to 0.08 in the first 30 s after spiking while it decayed to 0.11 in the seawater used for the multiple doses experiment. The equivalent chlorine demands are 0.12 mg/L in the single dose experiment and 0.09 in the multiple dose experiment. During the single dose experiment only one spike of chlorine was performed to achieve 0.1, 0.05 and 0.02 mg/L TRO. For the multiple doses experiment, spikes were performed to achieve concentrations of 0.02 and 0.05 mg/L TRO. In both cases, TRO was measured immediately after the spiking and after 30 min to assess decay. Results were highly variable due to the low concentrations selected which are close to the limit of detection for the method. A faster decay was observed during the multiple dose experiment with TRO concentrations detected only in some of the replicates for the 0.02 mg/L treatment group after 1 min from the first spike and after 30 min from the following two doses (Table 1).

Size distribution in both experiments changed at the highest concentrations tested with a loss of cells in the lower size classes (up to 4

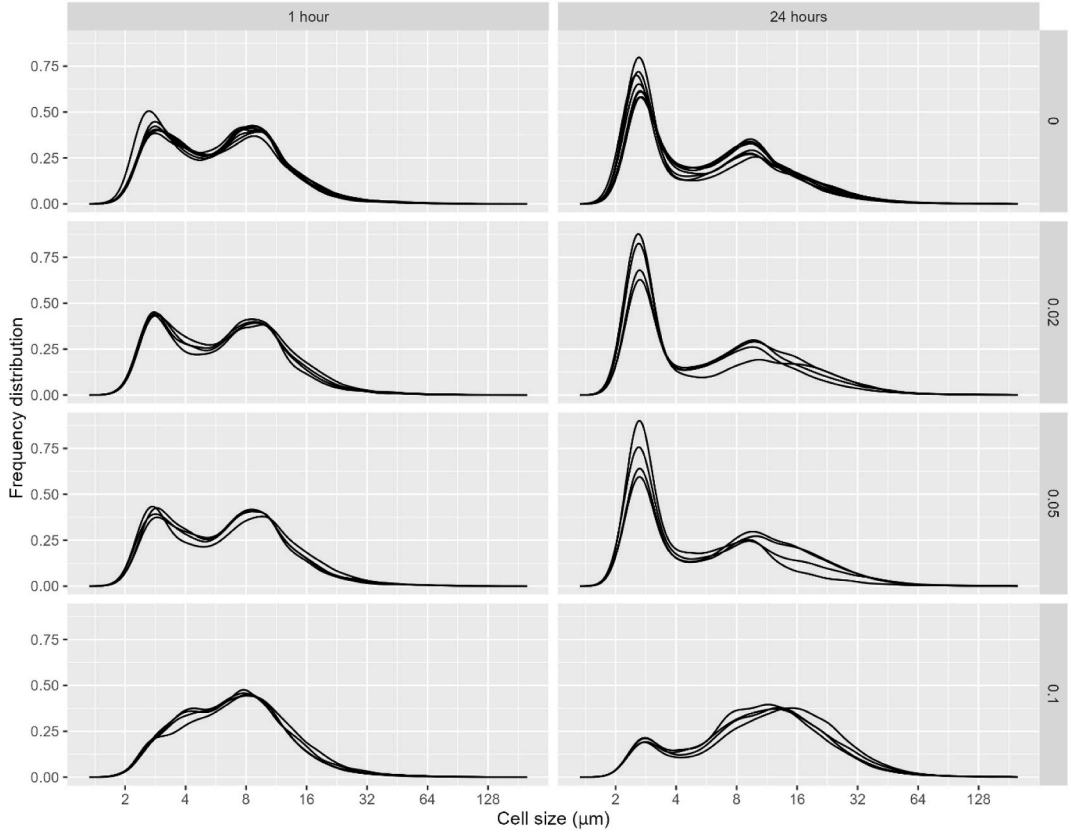

Fig. 1. Size distribution of a phytoplankton community exposed to increased TRO concentration (mg/L) as a single dose after 1 h (left column) and 24 h (right column). Concentration ranges are represented vertically. Multiple lines indicate replication in each treatment group. Forward scatter size data were log transformed and represent cells sizes ranging from 1 to 200 μm.

μm) and a consequent relative increase in the higher size classes (Fig. 1, single dose experiment; Fig. 2, multiple doses experiment). For the single dose experiment, the control group showed a bimodal distribution at both 1 h and 24 h. At 1 h the distribution showed two similar peaks, which represented 30–40% of the total cell numbers for control, 0.02 mg/L and 0.05 mg/L TRO groups. The highest treatment group (0.10 mg/L TRO), instead, showed a decrease to 20% for cells belonging to the size class 2–4 μm and a relative increase in the size class 8–16 μm which increased to 44% (ANOSIM R = 1, p = 0.003). At 24 h the biggest changes were observed for the highest treatment group (0.10 mg/L TRO, ANOSIM R = 1, p = 0.003) which showed a decrease in relative abundance to 14.87% for cells between 2 and 4 μm and a relative increase for the size groups 8–16 μm (43.17%) and 17–50 μm (27.25%) (see Table 2).

A different initial size distribution was observed during the multiple doses experiment with a unimodal distribution with a main peak for cells of size between 2 and 4 μm which represented 62% of the population in the control group. Significant changes were observed in both treatment groups. The biggest change was observed in the 0.05 mg/L treatment group with a reduction in cells numbers in the small size range (2–4 μm) to 40% of the total cell numbers and a relative increase to 32.9% of the size group 8–32 μm (ANOSIM R = 1, p = 0.002). Intermediate results were observed for the lower treatment group (0.02 mg/L TRO) with a reduction of the small size range (2–4) to 56% (ANOSIM R = 0.45, p = 0.013). Full ANOSIM results are shown in Table 2.

The abundance of functional groups in both the experiments showed a decrease in total cell numbers and a change in relative abundance in the highest treatment groups (0.10 mg/L TRO for the single dose experiment and at 0.05 mg/L TRO for the multiple dose experiment, Fig. 3).

In the single dose experiment, after 24 h the total number of cells in the control group was 17.5×10^4 cells/ml and was significantly reduced

Table 2
Results of ANOSIM analysis for both the experiment (single dosing and multiple dosing) at the relevant observed time points (1 h and/or 24 h) for results obtained with particle size distribution.

Experiment	Time (hours)	Group comparison	ANOSIM statistic R	Significance
Single dosing	1	Control vs 0.02	0.221	0.100
	1	Control vs 0.05	0.008	0.279
	1	Control vs 0.10	1	***0.003***
	24	Control vs 0.02	0.171	0.134
	24	Control vs 0.05	0.258	0.063
	24	Control vs 0.10	1	***0.003***
Multiple dosing	24	Control vs 0.02	0.4449	***0.013***
	24	Control vs 0.05	1	***0.002***

Table 3
Results of ANOSIM analysis for both the experiment (single dosing and multiple dosing) at the relevant observed time points (1 h and/or 24 h) for results obtained with flow cytometry clustering.

Experiment	Time (hours)	Group comparison	ANOSIM statistic R	Significance
Single dosing	1	Control vs 0.02	0.333	***0.034***
	1	Control vs 0.05	0.309	***0.046***
	1	Control vs 0.10	1	***0.002***
	24	Control vs 0.02	0.351	***0.024***
	24	Control vs 0.05	0.493	***0.006***
	24	Control vs 0.10	1	***0.002***
Multiple dosing	24	Control vs 0.02	0.369	***0.046***
	24	Control vs 0.05	1	***0.002***

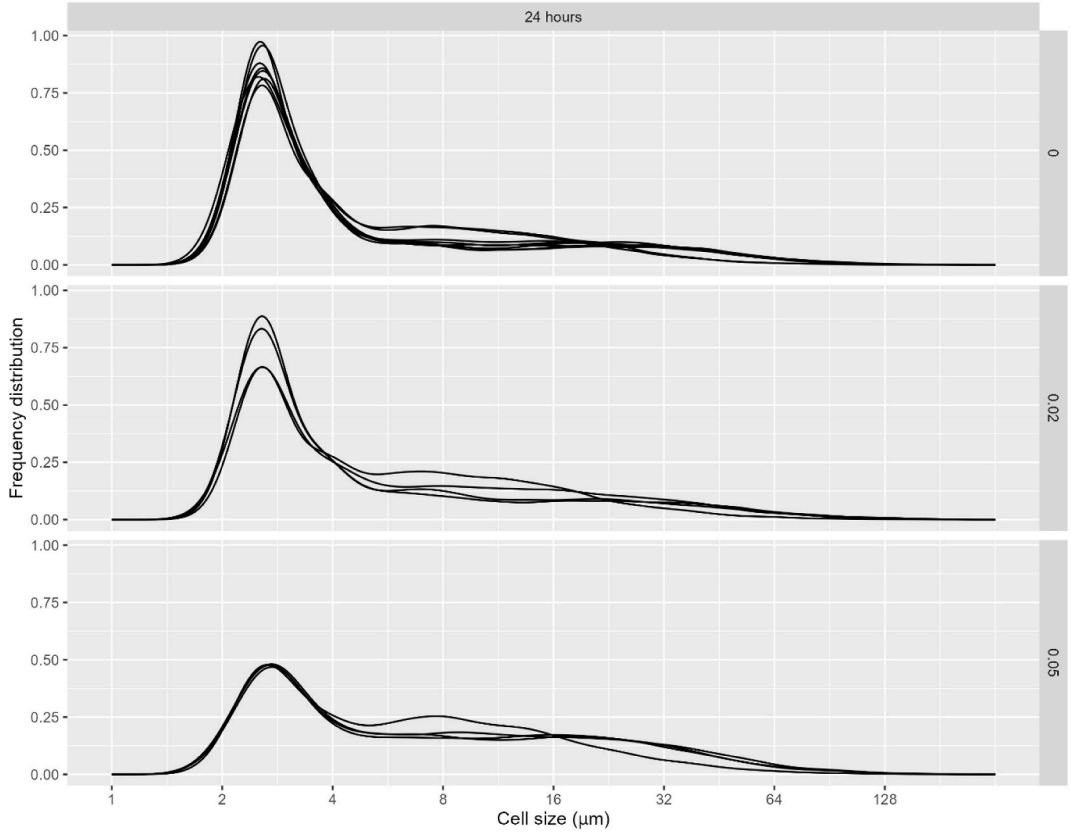

Fig. 2. Size distribution of a phytoplankton community exposed to multiple doses of TRO at increasing concentration (mg/L) after 24 h from the treatment. Concentration ranges are represented vertically. Multiple lines indicate replication in each treatment group. Forward scatter data were log transformed and represent cells sizes ranging from 1 to 200 μm.

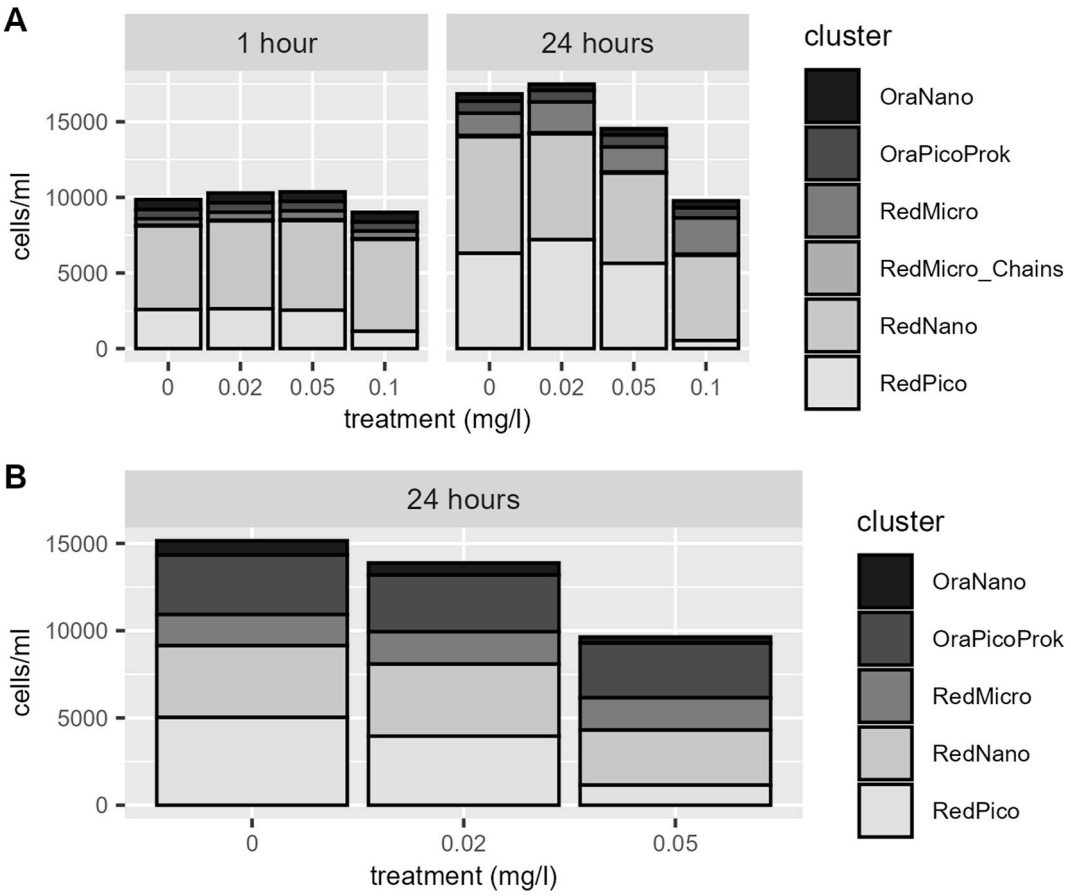

Fig. 3. Relative abundance calculated based on cells/ml for clusters obtained with flow-cytometry for the single dose experiment (A) and the multiple doses experiment (B). 1 h and 24 observations are included for the single dose experiment only (respectively left and right).

to 9.98 × 10^3 cells/ml in the 0.10 mg/L TRO treatment group ($p = 0.0128$). Red picophytoplankton was the main affected group at both 1 h and 24 h in the 0.10 mg/L treatment. This group decreased in relative abundance from 37.39% in the control group to 5.48% in the 0.10 mg/L TRO group at 24 h. In contrast, for the same time point, the red microphytoplankton increased from 8.76% in the control to 24.51% in the 0.10 mg/L TRO. The full ANOSIM analysis is summarised in Table 3 and raw cell numbers are included in supplementary materials (Table S6).

The multiple doses experiment showed a similar trend. There was a decrease in total number of cells in both the treatment groups with the red picophytoplankton being the most affected (Fig. 3, B). Total cells in

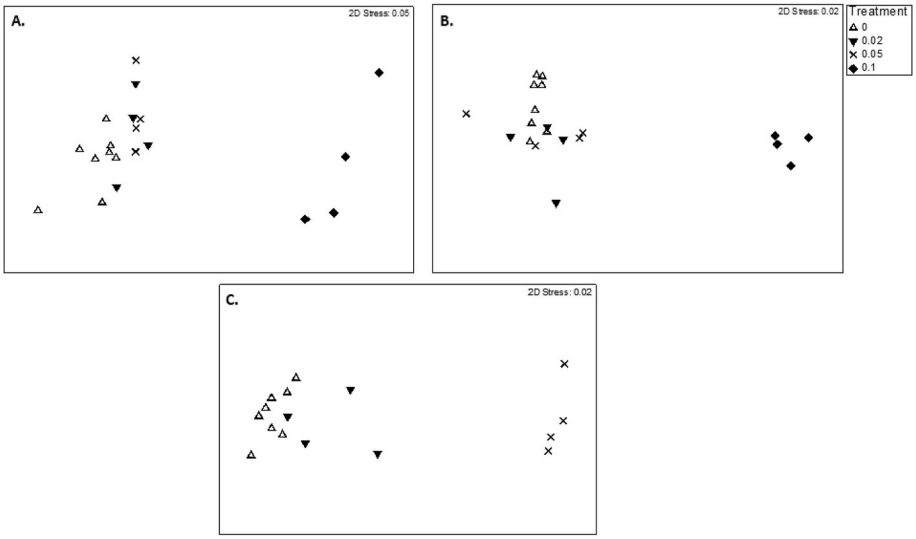

Fig. 4. Non-metric multidimensional ordination (nMDS) of functional diversity in phytoplankton communities during the two experiments obtained with flow-cytometry (n = 8 control and n = 4 treatment groups). Results are based on cell numbers standardised by total numbers and transformed using 4th root. Single exposure data 1 h after treatment (A.) and 24 h after treatment (B.); multiple exposure (24 h after treatment only, C.).

the control group were 1.62×10^4 cells/ml and significantly decreased to 1.03×10^4 in the 0.05 mg/L treatment group ($p = 0.00525$). In this case, the red picophytoplankton fraction was the only group showing a change in relative abundance with a decrease from 33.17% in the control group to 11.90% in the 0.05 mg/L treatment group (ANOSIM $R = 1$, $p = 0.002$). A significant change in the community functional types was also observed at 0.02 mg/L during the multiple doses experiment (ANOSIM $R = 0.369$, $p = 0.046$). For this treatment group, the red picophytoplankton component decreased to 25.77%.

PAM fluorescence results did not show significant differences and are included in supplementary materials (Fig. S3 and Table S3).

Multivariate analysis based on abundance of functional groups in the two experiments showed in both experiments separation of samples based on treatment (Fig. 4). For both experiments, and the two time points for the single exposure experiment, the highest chlorination treatment groups clearly separated from the rest. This is the case for 0.1 mg/L TRO treatment in the single dose experiment as it can be seen in Fig. 4 (A and B) and 0.05 mg/L treatment group (Fig. 4, C) ANOSIM analysis confirmed this separation showing statistical significant difference of these groups (ANOSIM $R = 1$, $p = 0.002$; . Statistical differences based on ANOSIM analysis were also observed in lower treatment groups although these were less significant (Table 3).

Carbon content, estimated on forward scatter size, did not show any trend with TRO concentration and results are shown in supplementary materials (Fig. S4). In the single dose experiment, at 1 h after the treatment, values varied between a minimum of 5.09 ng/L in the control group to a maximum of 6.96 ng/L in the 0.05 mg/L treatment group. After 24 h, values increased four times the highest value observed in the 0.02 mg/L treatment group with a value of 32.47 ng/L. Similarly for the multiple exposure experiment, values varied between 34.95 ng/L in the 0.05 mg/L treatment group and 47.78 ng/L in the 0.02 mg/L treatment group.

4. Discussion

The aim of this study was to evaluate the impact of secondary entrainment in the discharge plume of a power station upon natural phytoplankton communities. Flow cytometry was used as the main technique to evaluate potential impacts. A single exposure to TRO concentrations of up to 0.1 mg/L was chosen to represent a community entrained in the outer edges of the plume and a multiple exposure scenario to concentrations of up to 0.05 mg/L represented a community entrained in the plume and affected by tidal currents and as such re-entrained in this. These are in line with observed TRO discharges of a power station in the East of England (UK) in which the average concentration near the outfalls was 0.04 mg/L, up to a maximum of 0.16 mg/L (EDF Energy, 2020). Our study showed that there was no impact on total biomass in either of the evaluated scenarios, whether based on chlorophyll *a* or carbon content estimated from cell numbers and sizes. Community changes were instead observed with a decrease in cells belonging to eukaryotic picophytoplankton.

TRO decay in seawater is rapid and rates vary depending on water parameters such as temperature, salinity and organic matter (Jiangning et al., 2009; Turnpenny et al., 2010). Thus, it is likely that the different decay rates (and linked chlorine demand) observed here between the two experiments are a direct consequence of differences in seawater parameters that naturally vary, although these were not quantified in this study. Additionally, the two experiments showed differences in frequency distribution of the cells in the tested communities. In our case, these changes were observed between two sampling episodes at the same location with one month between visits in spring (April and June). This reflects that phytoplankton community composition can change over a short period of time as a reflection of environmental changes and nutrients availability (Lefebvre and Poisson-Caillault, 2019; Not et al., 2012).

Biomass based on carbon was estimated using cell size measured by flow cytometry, with the assumption that cells are spherical based on the relationship reported by Owen (2014). The same conversion was recently used to assess impacts of primary entrainment in cooling water systems on phytoplankton in Vannoni et al. (2021) which detected a significant reduction of biomass after chlorine treatment at a concentration of 0.2 mg/L (as TRO). In this study, biomass, based on carbon, did not decrease following chlorine treatment at any of the selected concentrations. Carbon concentrations estimated in this study are comparable to those reported for phytoplankton observed in the southwest of the UK (Capuzzo et al., 2022) indicating that the approach used here for the conversion can be representative of environmental scenarios (Fig. S4). Physiological changes based on variable chlorophyll fluorescence were not observed in either experiment (Fig. S3), in contrast with previous work using higher chlorination doses (0.2 mg/L) and temperature increase in an experiment to test the impact of primary entrainment on a phytoplankton community (Vannoni et al., 2021).

Community structure was impacted in both experimental scenarios, with a decrease in total number of RedPico phytoplankton (*sensu* Thyssen et al., 2022). This decrease was observed after both 1 h and 24 h in the single dose experiment indicating that there is an immediate impact of this group and no recovery after 24 h after 0.1 mg/L TRO dose. This is a polyphyletic group of photosynthetic cells <3 μm and includes the widely distributed Chlorophyta. Chlorophyta have been understudied because their small size means that they cannot be monitored by standard microscopy (Worden et al., 2004). In global metagenomic dataset they are the second most abundant group of photosynthetic Eukaryotes after Ochrophyta (predominantly diatoms) (Tragin and Vaulot, 2018). Amongst Chlorophyta, Mamiellophyceae dominated in terms of both number of reads and diversity (number of operational taxonomic units, OTUs) reaching up to 99% of Chlorophyta the North Sea. The genus *Ostreococcus* and *Micromonas* were identified in the Western English Channel (Romari and Vaulot, 2004) and in the North Sea (Tragin and Vaulot, 2019). Cyanobacteria (OraPicoprok from flow cytometry clustering, mainly represented by *Synechococcus*) were not impacted in any of the tested concentrations. The reduction in abundance in RedPico phytoplankton alone was sufficient to cause dissimilarity between treatments based on non-metric multidimensional clustering and confirmed by ANOSIM analysis.

Accessory pigments can be used to qualitatively and quantitatively assess phytoplankton diversity (Roy et al., 2011) and thus provide more detailed information on the taxonomic groups responsible for the changes seen in the flow cytometry data. They were quantified in a limited number of replicates in our study (see results in supplementary materials, Table S1). Prasinoxanthin and chlorophyll *b*, markers specifically present in various class of Chlorophyta (Roy et al., 2011; Worden and Not, 2008) decreased in the treatment groups. This agrees with the decrease in RedPico phytoplankton providing further evidence that Chlorophyta are the most sensitive component of these communities. The reason behind different sensitivity of different groups of similar size to chlorination remains unclear, although this may be due to smaller cells having a larger surface: volume ratio. Higher sensitivity of smaller cell species to polycyclic aromatic hydrocarbons was observed by Echeveste et al. (2010) although cyanobacteria were more sensitive than eukaryotic picophytoplankton, unlike what found here. Resilience to chlorination was observed for a microphytobenthos biofilm dominated by diatoms (Vannoni et al., 2022) which led to preliminary conclusions that extracellular polysaccharides produced by certain groups such as diatoms (Shniukova and Zolotareva, 2015; Wustman et al., 1998) and cyanobacteria (Kehr and Dittmann, 2015) provide protection against chlorination by creating a protective chemical barrier, due to the reaction of chlorine with the polysaccharide matrix. This was also observed for the bacteria *Pseudomonas aeruginosa* in which the authors demonstrated that EPS acts as chlorine consumer (Xue et al., 2013), thus, protecting the cells. In contrast to our findings, the cyanobacteria *M. aeruginosa* was more sensitive than the Chlorophyta *C. vulgaris* (Cao et al., 2021). These experiments were run in freshwater; thus, it is

possible that the inverse sensitivity found in our study may be due to the different chemistry of chlorine in seawater. Different sensitivity to chlorine was also found within the diatoms group with *Achnanthes* spp. being more sensitive than *Navicula pelliculosa* and EC_{10}s respectively of 0.02 and 0.04 mg/L TRO under continuous dosing for 72 h (Vannoni et al., 2018). Nevertheless, despite the similar size, cyanobacteria and other picophytoeukariotes fulfil different ecological functions (Worden and Not, 2008). Cyanobacteria are thought to be weaker than other groups in sustaining food webs due to the lack of essential biomolecules together with their small size (Schmidt et al., 2020). *Ostreococcus* and *Micromonas* were recently identified as keystone taxa in a 6 year study of coastal and offshore microbial communities of the Bay of Biscay, France (Garate et al., 2022). Therefore, the wider ecological impact of a potential reduction in the Mamiellophycea should be further investigated.

An assessment of available reliable toxicity data for the application of EQS highlighted a lack of good quality data for marine microalgae with a 24 h LC_{50} for *Thalassiosira pseudonana* of 0.075 mg/L as the only relevant data. Nevertheless, this value was regarded as not reliable due to the lack of verification of chlorine concentrations in the test solutions (Sorokin et al., 2007). Community data available for freshwater periphyton showed greater sensitivity with no effects detected at 3 μg/L TRC after 7 days. This value was obtained in a flow-through study where chlorine concentrations were maintained constantly (Cairns et al., 1990). This study fills a gap in data on algal groups highlighting high sensitivity of smaller size groups belonging to picophytoeukaryotes. The ecological significance of the decrease in relative abundance for this group is difficult to estimate due to lack of widespread research on this smaller component of the community. Nevertheless, studies show that picophytoplankton can be dominant in oligotrophic waters globally (Visintini et al., 2021). However, with the decrease in nutrient load in coastal waters in recent years, a shift in size composition of phytoplankton towards smaller cells has been observed (Holland et al., 2023; Schmidt et al., 2020). Consequently, recommendations were made to include picophytoplankton in phytoplankton monitoring programmes (OSPAR, 2016) and to be used as ecosystem health indicators in EU Marine Strategy Framework (MSFD) and UK Marine Strategy (McQuatters-Gollop et al., 2024). In general, limited data is available on sensitivity of picophytoplankton groups to chemicals, and standard ecotoxicological testing guidelines do not include these groups. Picophytoeukaryotes were found to be the most sensitive amongst picophytoplankton taxa to PAHs (Ashok and Agusti, 2022) and picophytoplankton as a total group was the most sensitive group to higher chlorination doses (Vannoni et al., 2021) highlighting the importance of including this group in routine assessments of chemical risks.

The EQS for chlorine in seawater is based on high limits of detection for current analytical methods rather than on the most sensitive toxicity data. Although this is a current limitation, modelling data for discharge plumes could be used to understand expected concentrations of chlorine in the environment. Further studies should focus on standard toxicity tests with species belonging to picophytoeukaryotes and using sublethal endpoints which would be valuable to refine current EQS for chlorine. This work demonstrates that phytoplankton community studies and flow cytometry are effective tools to determine the potential impacts of chemical discharges from anthropogenic activities in the marine environment.

5. Conclusions

To the best of our knowledge this is the first study examining the effects of low chlorine doses (<0.1 mg/L) on phytoplankton communities or marine microalgae in the laboratory. Despite changes in seawater characteristics and community assemblages between the two experiments, similar results were observed. Our overall results suggest that chlorine at doses up to a maximum of 0.1 mg/L cause limited impacts. There was no evidence of impact on the total biomass (based on estimated carbon). Nevertheless, a shift in community composition based on flow cytometry clustering was observed in both experiments at the highest concentrations tested due to a reduction in abundance in picophytoeukaryotes which were the most sensitive group. These effects are expected to be localised in the area surrounding cooling water discharges. Chlorination has other applications in desalination plants and ballast waters treatment and as such the potential impact of the decrease in this group on the food webs and biogeochemical cycles should be further investigated. Refinement of EQS for chlorine is also recommended to consider the most sensitive taxa.

Flow cytometry was shown to be the most appropriate technique to detect small changes in functional group of phytoplankton communities. Standard methods normally used for phytoplankton communities monitoring, such as microscopy and chlorophyll *a*, overlook smaller cell classes, are more expensive, time consuming and require bigger sample volumes. Additionally, flow cytometry, with its small variation between replicate samples allowed the detection of much smaller changes in the community. The use of community studies and multiple approaches can help the understanding of how the whole community is affected by anthropogenic activities in a more comprehensive way than more standard ecotoxicology methods. These studies should be considered as complementary to standard toxicity studies in assessing chemical risks. Furthermore, this study highlighted that picophytoplankton, which includes important keystone species and is indicative of community health, is the most sensitive group to chlorination in a natural phytoplankton community. For this reason, species belonging to this group should be included in regulatory toxicity testing for the marine environment.

CRediT authorship contribution statement

Marta Vannoni: Writing – review & editing, Writing – original draft, Investigation, Formal analysis, Data curation, Conceptualization. **Alastair Grant:** Writing – review & editing, Supervision. **Dave Sheahan:** Writing – review & editing, Supervision, Project administration, Methodology, Conceptualization. **Véronique Créach:** Writing – review & editing, Supervision, Methodology, Investigation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2024.143634.

Data availability

Data will be made available on request.

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