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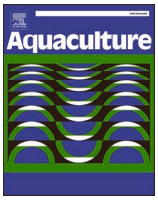

Short communication

Supplementing air with CO_2 stripped from recirculating aquaculture improves growth of two green microalgae in aquaculture wastewater

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ABSTRACT

To improve sustainability and to implement the principles of circular economy in aquaculture, we tested the possibility to boost the capture of nitrate by two green microalgal species from recirculating aquaculture system's (RAS) wastewater by supplementing air with carbon dioxide stripped from a RAS. Carbon dioxide addition increased cell densities of *Monoraphidium griffithii* and *Haematococcus pluvialis* in photobioreactors during 9-day growing periods. However, growth rates and nitrate uptake rates were only improved for *M. griffithii*. Addition of CO_2 decreased pH of the medium with *M. griffithii* which likely also affected positively on algal growth and nutrient uptake. These laboratory scale experiments suggest that microalgal cultivation to produce valuable biomass could be connected to a RAS to decrease nitrate and CO_2 emissions from aquaculture.

1. Introduction

A recirculating aquaculture system (RAS) is a technology used to support intensive aquaculture production on land, especially fishes, with limited use of water. Although covering only a small fraction of the total aquaculture production within EU (Bostock et al., 2016), RAS has gained popularity during the last decade (Bostock et al., 2016; Eumofa, 2021) due to the possibility for strict control of the system and continuous production under stable environmental conditions. In RAS, the water is passed through different technical installations to remove solids, to transform ammonia released by the fish into less harmful nitrate, to strip excess CO_2, and to kill pathogens, before pumping the water back to the fish tanks. During recirculation the concentration of dissolved nutrients increases in the system and this increase is commonly controlled by diluting the system with fresh water. The RAS effluent typically contains high concentration of dissolved nutrients, e.g. nitrate up to 100 mg/l (Davidson et al., 2014) and phosphate up to 45 mg/l (van Bussel et al., 2013) have been reported, but instead of disposing the nutrient-rich water into natural water courses or water treatment plants, it could be further used for growing plants (Goddek et al., 2019) or microalgae (Stevčić et al., 2019; Ramli et al., 2020).

Fish and bacterial respirations in a RAS increase the water CO_2 concentration and it must be stripped before pumping the water to fish tanks to avoid the potential negative effects of high CO_2 concentration on fish (Mota et al., 2019; Skov, 2019). To increase the sustainability and to decrease the carbon footprint of RAS-produced fish, it would be ideal to use not only the dissolved nutrients but also the excess CO_2 for production of photoautotrophic organisms. Microalgae could serve as an option for this purpose, as microalgal growth can be stimulated by moderate (up to 5%) CO_2 addition (Chekanov et al., 2017). It has also been suggested that by pumping room air through microalgal bioreactors they could serve as room air filters (Cheng et al., 2006). As the RAS are practically always built indoors, it would be beneficial to filter CO_2-rich air from CO_2 strippers with microalgae: first, it would decrease the energy need for ventilation (important especially in colder climate zones in winter when outdoor air must first be heated in the ventilators) and second, it could increase biomass production in algal bioreactors.

There is a lot of information available separately on the use of different types of wastewaters for producing microalgae through bioremediation (Christenson and Sims, 2011; Li et al., 2019; Chai et al., 2021), as well as of the effects of addition of CO_2 to algal cultures (Goli et al., 2016; Neves et al., 2019). However, there appears to be very little information available on the combination of nutrient and CO_2 capture from wastewater using microalgae (Molazadeh et al., 2019). In previous experiments, we found that green microalgae (Chlorophycae) are suitable candidates to be cultivated in RAS wastewater at a relatively low temperature (~17 °C) (Stevčić et al., 2019; Calderini et al., 2021). Here we tested if the growth and nutrient uptake of two previously tested species, *Monoraphidium griffithii* and *Haematococcus pluvialis,* could be further enhanced when cultivated in RAS wastewater supplemented

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with CO_2 stripped from a RAS. Our hypothesis was that CO_2 supplementation would increase microalgal cell density and their NO_3-N uptake.

2. Materials and methods

Two separate experiments were carried out between April – June 2021 in the laboratory of the department of biological and environmental science, University of Jyväskylä, Finland. The algal species used were *M. griffithii* (strain: NIVA-CHL 8, Norway) and *H. pluvialis* (K-0084 (NIVA), Sweden). The algae were cultivated in photobioreactors, which consisted of transparent plastic funnels (total volume 1.5 l). Ten funnels were attached in one row, about 2.5 cm apart and covered with transparent plastic lids to avoid excess evaporation and to decrease the likelihood of contamination. Every other funnel ($n = 5$) received room air from below with an air pump through an air stone (on average 500 ml/min), and every other (n = 5) was connected to an air pump located in an airtight plastic bag receiving CO_2 rich air from an experimental size RAS (total volume about 4.5 m^3) trickling filter (Fig. 1), which was used to strip CO_2 from water and to add O_2. One side of the funnel was illuminated constantly with a LED light (18 W, AP67 T8 tubes, Valoya Oy, Finland) with light intensity of c. 100 μE m^{-2} s^{-1}. The water used in the photobioreactor originated from another experimental size RAS (total volume about 750 l), housing rainbow trout (*Oncorhynchus mykiss*), and it was passed through a 48 μm mesh before use. Temperature in the photobioreactors varied between 17 and 18 °C. These rearing conditions were similar in both experiments.

Algal density was quantified daily by counting on a haemocytometer (Bürker) under a microscope with 100× magnification. Water temperature and NO_3-N concentration were measured with YSI Quatro multiprobe meter (Yellow Spring Instruments, USA) and pH with Eutech PC 450 (Thermo Scientific Eutech, Singapore). The incoming air CO_2 concentration was measured with AirControl COACH CO2- Monitor (Dostmann electronic GmbH, Germany) from empty funnels connected to air pumps from lab air or RAS trickling filter air.

M. griffithii was cultured in batch mode (all medium added in the beginning) with 150 ml of algal suspension added into 850 ml of RAS wastewater (filtered through a 48 μm mesh), equalling to a 15% inoculum volume to total volume ratio and an initial concentration of 1.3 × 10^5 cells/ml (day 0). The growth of the algae was monitored for 10 days. During the experiment, average room air CO_2 concentration was 527 ppm (0.0527%; min 497 – max 563 ppm) and in the air pumped from the RAS, 985 ppm (0.0985%; 910–1026 ppm). *H. pluvialis* was cultured in fed batch mode (medium added at pre-defined intervals), with initial addition of 65 ml of algal suspension into 340 ml of RAS wastewater in vegetative green phase (see e.g. Shah et al., 2016, for details of *H. pluvialis* life cycle), equalling to a 16% inoculum volume to total volume ratio and an initial concentration of 2.8 × 10^4 cells/ml (day 0). 405 ml of wastewater was further added on days 4 and 7 (total volume 1215 ml). Average room air CO_2 concentration was 526 ppm (range 493–575 ppm) and in the air from RAS it was 925 ppm (805–1166 ppm) (Fig. 2).

Growth rate (GR, d^{-1}) was calculated for each culture as $(\mathrm{Ln}N_2 - \mathrm{Ln}N_1) * t^{-1}$, where N_1 and N_2 were concentrations of microalgae (ml^{-1}) in the beginning and end of the visually estimated exponential growth period t, respectively. For *M. griffithii* the period t was nine days. For *H. pluvialis* we calculated GR separately for three periods matching the addition of wastewater: days 1–3, days 3–5, and days 5–6 (see Fig. 1b), and then calculated the average GR for each culture to be used in the statistical analyses. Statistical analyses were done with SPSS version 26 (IBM SPSS Statistics). Possible differences between the treatments (air vs. CO_2 supplement) in SGR were compared with independent samples t-test as the sample variances did not differ (Levene test). Differences in cell density, nitrate concentration and pH-value were analysed by comparing daily means of the treatments with GLM repeated measures ANOVA. Day was included as a within-subject factor and treatment as a between-subject factor. The sphericity assumption was tested with Maulchy's test, and if the sphericity was violated, Greenhouse–Geisser corrected values were used. Normality of data was tested with Kolmogorov–Smirnov test, but the assumption was not always met. Results of rmANOVA are reported, as the risk of false positive result is not much affected by violation of normality for ANOVA (Lix et al., 1996). For the post-hoc comparisons Bonferroni corrected values were used. A value of $p < 0.05$ was used as the level for statistical significance.

3. Results and discussion

Carbon dioxide supplementation increased cell densities (Fig. 3; *M. griffithii* rmANOVA, $F_{1,8} = 22.759$, $p = 0.001$ and *H. pluvialis* rmANOVA, $F_{1,8} = 22.935$, $p = 0.001$). In addition, the interaction between day and treatment was significant with both algae (*M. griffithii*: rmANOVA, $F_{2.3} = 8.368$, $p = 0.002$, *H. pluvialis*: rmANOVA, $F_{3.7} = 3.164$, $p = 0.031$), showing that CO_2 addition increased algal densities during the experiment. Average ± S.D. growth rate over nine days for *M. griffithii* were 0.43 ± 0.01 day^{-1} and 0.48 ± 0.02 day^{-1} in cultures without and with CO_2 supplementation, respectively ($t_8 = 4.23$, $p = 0.003$) and for *H. pluvialis* the respective values were 0.44 ± 0.08 and 0.52 ± 0.16 ($t_8 = 1.26$, $p = 0.24$). For *H. pluvialis* growth rate and densities were similar when compared to earlier results in our laboratory in batch culture (Stevčić et al., 2019) or in experiments where different types of growth media and environmental conditions have been tested (Gong and Chen,

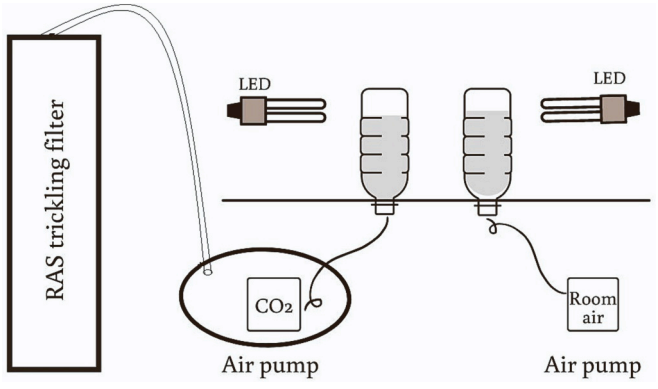

Fig. 1. A schematic presentation of the setup where the growth of microalgae was compared between funnels receiving either room air or CO_2-rich air stripped from a trickling filter of a 4.5 m^3 Recirculating Aquaculture System (RAS). Funnels (5 replicates in both treatments) were filled with 1.2 l of RAS wastewater. Air pumps for the CO_2 treatment were in an airtight plastic bag.

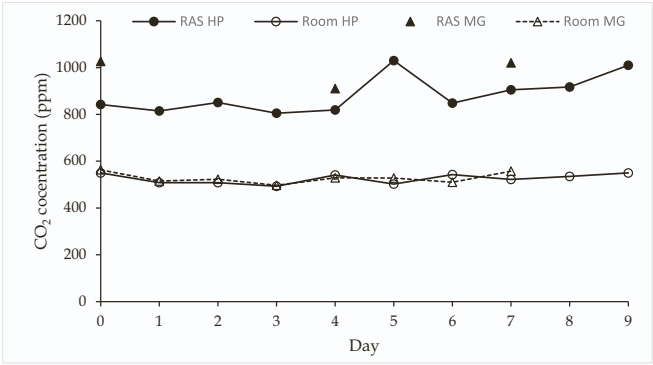

Fig. 2. Concentration of CO_2 in room air (open symbols) and air stripped from a Recirculating Aquaculture System (RAS, filled symbols) in two growth experiments with green microalgae. The first experiment was done with *Monoraphidium girffithii* (MG) and the second with *Haematococcus pluvialis* (HP).

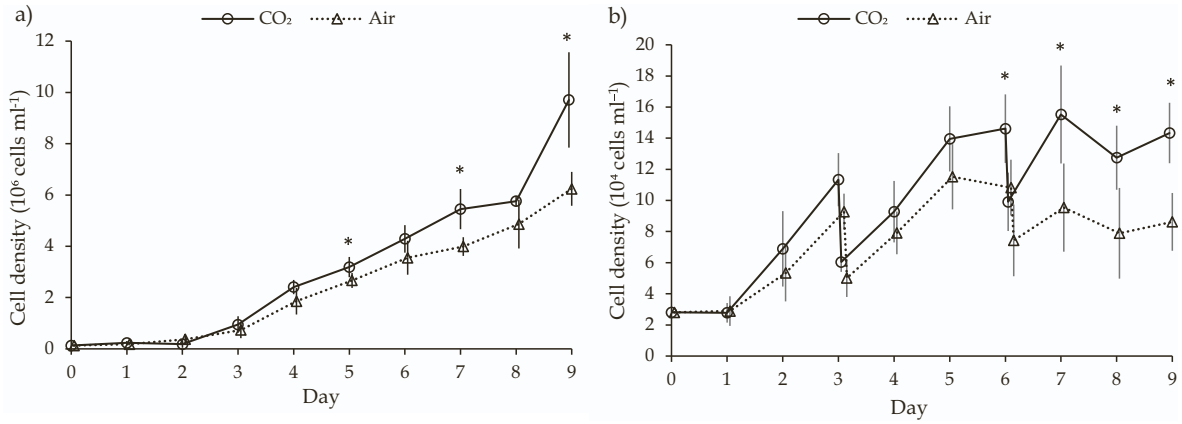

Fig. 3. Average (±SD, $n = 5$) algal density in photobioreactors with a) *Monoraphidium griffithii* and b) *Haematococcus pluvialis* aerated with room air (triangle, dotted line) or CO_2 supplemented air pumped from a trickling filter of a RAS (circle, solid line). *M. griffithii* was cultivated in batch mode, *H. pluvialis* with fed-batch mode, where 1/3 of the medium (RAS wastewater) was added on days 3 and 6, and measurements were taken before and after the addition. Asterisks indicate statistical difference ($p < 0.05$) between the two treatments. Note the different scales on y-axes.

1997; Kaewpintong et al., 2007). For *M. griffithii* growth rate and densities were also comparable to the experiments conducted earlier with RAS wastewater in our laboratory (Stevčić et al., 2019; Calderini et al., 2021). The similarity of growth rate in the present experiments to those reported earlier demonstrates that the growth conditions in our experiment were suitable for testing the effect of CO_2 addition for these algae.

Nitrate is one of the most important nutrients supporting the growth of microalgae (Wang et al., 2019; Nur et al., 2021), and nitrate is available in high concentrations, up to 100 mg/l (Davidson et al., 2014), in RAS wastewater. Therefore, by combining microalgal cultures to RAS farming, the environmental effects of fish farming could be decreased along with the production of algal biomass. Even if the water consumption in intensive RAS farming is typically reduced over 90% as compared to flow-through farming (Murray et al., 2014), the volume of the effluent can still be large. Taking into account the time required for algal biomass growth and their requirement for light, algal photobioreactors have a limited capacity for purification of large effluent volumes. Thus, methods to increase algal growth and nutrient uptake from RAS wastewater should be developed to intensify the water purification process. In this experiment cultures receiving additional CO_2 stripped from the RAS had a significantly lower nitrate concentration in wastewater when *M. griffithii* was used (Fig. 4a; rmANOVA, $F_{1,8} = 26.848$, $p = 0.001$) but with *H. pluvialis* the average nitrate concentration was not lower in CO_2 treatment (Fig. 4b; rmANOVA, $F_{1,8} = 3.168$, $p = 0.113$). It seems that the possible increase in nutrient uptake along with the increase of CO_2 concentration may depend on the microalgal species. Nutrient uptake (both ammonium and nitrate) was reported to increase with green microalga *Desmodesmus communis* when aeration was supplemented with 2% CO_2 (Pezzolesi et al., 2019). On the other hand, CO_2 addition (0–20%) did not have significant effect on nutrient (ammonium, nitrite, nitrate, phosphate) removal efficiency when *Chlamydomonas acidophila* was used even if the algal productivity increased at CO_2 concentrations 5% and 10% (Neves et al., 2019).

CO_2 supplementation decreased pH in cultures with *M. griffithii* (Fig. 5a; rmANOVA, $F_{1,8} = 25.716$, $p < 0.001$) but not in *H. pluvialis* cultures (Fig. 5b; rmANOVA, $F_{1,8} = 5.032$, $p = 0.055$). A lower pH in CO_2 treated cultures was expected as CO_2 addition is known to decrease pH, and CO_2 addition is also used to maintain pH at a desired level in photobioreactors (Pedersen et al., 2018). In *M. griffithii* cultures, a significant drop in pH was observed on day two (Fig. 5a). Similar drop and subsequent increase in pH has been reported previously with *Chlorella vulgaris* and *Chlamydomonas reinhardtii* (Scherholz and Curtis, 2013), and explained with the preference of the algae to use ammonia over nitrate (Pezzolesi et al., 2019) as ammonia uptake releases protons causing a decrease in pH. On the other hand, the increase of pH is most likely linked to photosynthesis which is known to induce build-up of

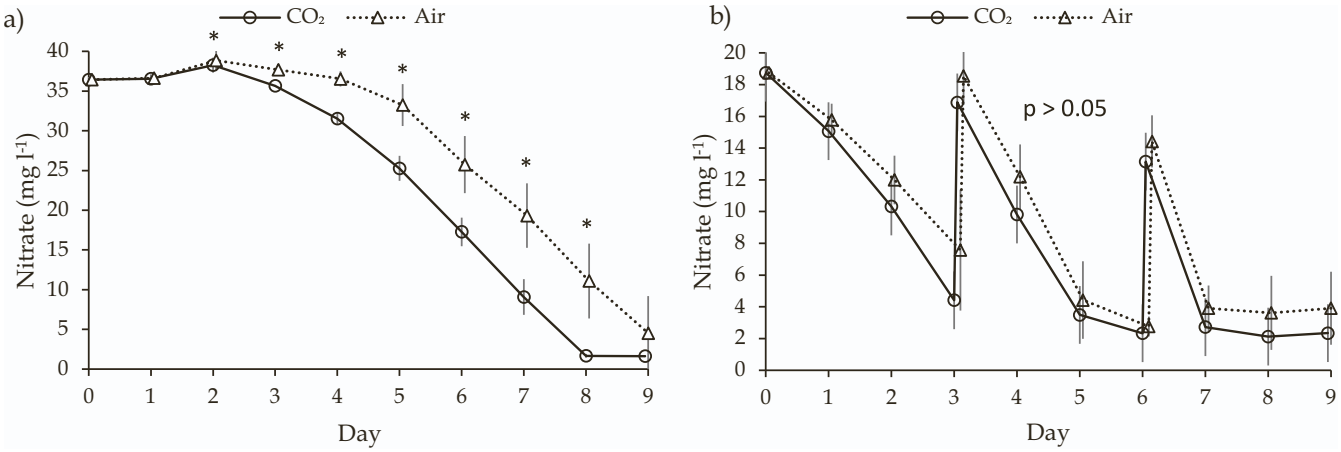

Fig. 4. Average (±SD, $n = 5$) water nitrate (NO_3-N) concentration in photobioreactors with a) *Monoraphidium griffithii* and b) *Haematococcus pluvialis* aerated with room air (triangle, dotted line) or CO_2 supplemented air pumped from a trickling filter of a RAS (circle, solid line). *M. griffithii* was cultivated in batch mode, *H. pluvialis* with fed-batch mode, where 1/3 of the medium (RAS wastewater) was added on days 3 and 6, and measurements were taken before and after the addition. Asterisks indicate statistical difference ($p < 0.05$) between the two treatments.

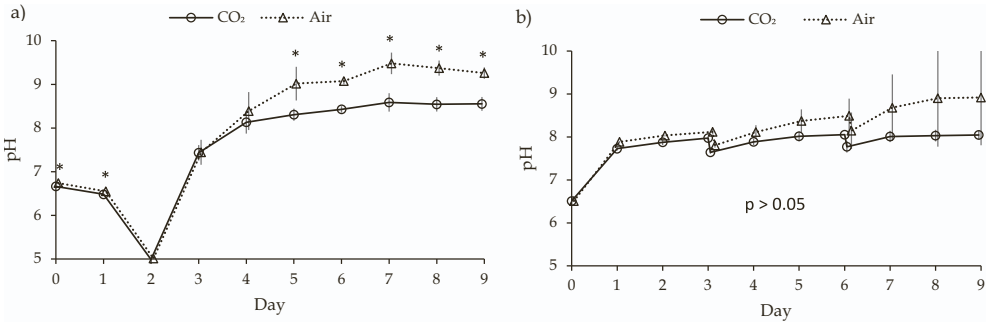

Fig. 5. Average (±SD, $n = 5$) water pH in photobioreactors with a) *Monoraphidium griffithii* and b) *Haematococcus pluvialis* aerated with room air (triangle, dotted line) or CO_2 supplemented air pumped from a trickling filter of a RAS (circle, solid line). *M. griffithii* was cultivated in batch mode, *H. pluvialis* with fed-batch mode, where 1/3 of the medium (RAS wastewater) was added on days 3 and 6, and measurements were taken before and after the addition. Asterisks indicate statistical difference ($p < 0.05$) between the two treatments.

OH^- and CO_2 uptake, as well as to the nitrate assimilation (Larsdotter, 2006; Geada et al., 2017). As the optimal pH for cultivation of *M. griffithii* and *H. pluvialis* is close to neutral (Sarada et al., 2002; Fujii et al., 2008), CO_2 addition caused a dual positive effect on the growth of both algae: first, it made the pH more favourable for the algal growth, and second, it provided the algae with inorganic carbon to allow for increase in growth rate.

In the CO_2 treatment, CO_2 concentration pumped into the cultures from the RAS trickling filter was around 900–1000 ppm. Summerfelt et al. (2000) reported that air CO_2 concentration in a cascade column filter outlet varied between c. 1000 and 8400 ppm depending on the packing depth of the stripping column, gas-to-liquid (i.e. air to water) ratio and water CO_2 concentration, resulting in an increase of the air CO_2 concentration before and after the filter from c. 70 to 500% (Summerfelt et al., 2000). In our system the increase of CO_2 concentration between the inlet (room air pumped into the trickling filter) and outlet (from the filter) air was on average from 75% (the experiment with *H. pluvialis*) to 86% (*M. griffithii*). This relatively low increase in CO_2 concentration was most likely due to low fish density (less than 5 kg/m^3) in our RAS. However, rainbow trout could be reared in densities between 50 and 100 kg/m^3 in RAS (Roque d'Orbcastel et al., 2009). In such intensive settings CO_2 concentration of the air from the degasser could be expected to be much higher than in the present experiment, and consequently also the growth of the algae could potentially be further increased.

Different types of wastewaters have been used for producing microalgae (Larsdotter, 2006), and CO_2 in industrial flue gases have been used to boost the growth of microalgae. At the same time microalgae serve as biological cleaners of wastewater, and they assimilate the greenhouse gas CO_2 (Geada et al., 2017). In aquaculture, especially on land-based RAS, microalgae could be used to capture both the dissolved nutrients and CO_2 to mitigate their environmental effects and to promote circular economy in aquaculture. In contrast to many other types of wastewaters and flue gases, RAS wastewater and CO_2 are void of metals or other detrimental pollutants that could limit the use of microalgae also for other purposes than biodiesel production (Goswami et al., 2021). In conclusion, the current results can be regarded as a proof of concept, suggesting that the growth of microalgae grown in RAS wastewater can easily be increased by using CO_2 stripped from a RAS, thus giving an option to make aquaculture production more sustainable and environmentally friendly. Of the two tested algal species *M. griffithii* appeared to be more responsive than *H. pluvialis* in terms of growth rate and nutrient uptake to the addition of CO_2. The experiments were done in laboratory scale with a relatively low concentration of nitrate and CO_2 and thus, further experimentation would be needed in a setting comparable to commercial farming, which could be expected to increase algal growth rate and nutrient uptake. This laboratory scale experiment does not permit us to make reasonable estimates of overall efficiency of this type of integrated system in commercial fish farms. Pilot-scale experiments would be needed to make an approximate estimate of commercial feasibility of such a system, and the profitability will depend very much on the end-use of the selected algae. However, our system indicates that integration of microalgal cultures with RAS is an option towards greener and ecologically sustainable aquaculture.

CRediT authorship contribution statement

Juhani Pirhonen: Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing – original draft, Supervision, Project administration. **Silja Koukka:** Formal analysis, Investigation, Data curation, Writing – review & editing, Visualization. **Katja Pulkkinen:** Conceptualization, Methodology, Formal analysis, Investigation, Resources, Data curation, Writing – review & editing, Supervision.

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.

Data availability

Data will be made available on request.

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