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Changes in long chain alkenone distributions and Isochrysidales groups along the Baltic Sea salinity gradient

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abstract

Isochrysidales species of the phylum Haptophyta are the exclusive producers of C_{37} to C_{42} long chain alkyl ketones, also called long chain alkenones (LCAs). While LCA distributions are known to vary with temperature and salinity, it is difficult to tease apart the direct effects of environmental parameters vs changes in the LCA-producing organisms. The Baltic Sea surface salinity gradient, which ranges from oligohaline (0.5–5 g/kg) to polyhaline (18–30 g/kg), represents a unique opportunity to study the relationships between salinity changes, species distribution and LCA biomarkers in a single ecosystem. LCA biomarkers revealed the presence of the three known Isochrysidales groups (Groups I, II and III) in Baltic Sea surface sediments, and the presence of Groups I and II were further confirmed with DNA sequencing. Group III Isochrysidales were present in the mixoeuhaline Skagerrak based on LCA signature alone. Groups I and II Isochrysidales were found for the first time in the Baltic Sea using a combination of LCAs and DNA biomarkers, solving an eighteen-year long mystery of Baltic Sea LCA-producing haptophyte identity. Group II Isochrysidales, which have a large salinity tolerance range, were spread over the Skagerrak and the complete Baltic Sea, but were characteristic for the central Baltic Sea. Oligohaline Group I Isochrysidales were representative for the northern Baltic Sea. However, evidence of Group I Isochrysidales in the central and southern Baltic Sea suggests a possible transport by surface currents since this group is typically confined to oligohaline conditions. Testing the recently developed ratio of isomeric C_{37} ketones (RIK_{37}) against the Baltic Sea surface salinity gradient revealed a significant positive correlation. This may represent a salinity proxy reflecting the amount of Group I Isochrysidales relative to Group II Isochrysidales in oligohaline environments. The present study elucidates for the first time the identity and the spatial distribution of LCA producers thriving in a large and stable brackish environment.

1. Introduction

Haptophytes are single-celled phytoplankton that have a worldwide distribution and are one of the major groups of primary producers in the ocean, together with cyanobacteria, diatoms, dinoflagellates and prasinophytes (Liu et al., 2009; Edvardsen et al., 2016). Members of the order Isochrysidales are unique in that they are the only known organisms producing LCAs, alkyl ketones with 35–42 atoms of carbon, two to four double bonds, a methyl or ethyl configuration and tri-unsaturated isomers (de Leeuw et al., 1980; Volkman et al., 1980; Farrimond et al., 1986; Rontani et al., 2004; Longo et al., 2013, 2016; Zhao et al., 2014). Examples of cultured long chain alkenone (LCA) producing species include Emiliania huxleyi, Gephyrocapsa oceanica, Isochrysis galbana, Ruttnera lamellosa and Tisochrysis lutea. However, in the recent decade, environmental molecular ecology studies of lacustrine environments have revealed the presence of many other phylotypes of the Isochrysidales order that are potential LCA-producing species (D'Andrea et al., 2006; Theroux et al., 2010; Toney et al., 2012; Simon et al., 2013; Randlett et al., 2014; Egge et al., 2015; Longo et al., 2016).

Recent publications suggest that there are three main groups (Group I, II and III) of LCA-producing Isochrysidales (Theroux et al., 2010; Longo et al., 2016). Group III Isochrysidales is

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represented by E. huxleyi and G. oceanica strains found only in the marine environment. Group II Isochrysidales includes organisms genetically related to Isochrysis spp., R. lamellosa, and T. lutea, found in marine (coastal) and lacustrine environments. Finally, Group I Isochrysidales, more recently discovered (D'Andrea et al., 2006; Theroux et al., 2010; Simon et al., 2013), comprises exclusively uncultured haptophytes that are known only from lacustrine environments. These phylotypes occupy different salinity ranges. While Group III Isochrysidales occur in mesohaline to mixoeuhaline waters (11–41 g/kg; Winter et al., 1994), Group II Isochrysidales occur in oligohaline to hyperhaline waters (>0.5 g/kg; Kaplan et al., 1986; Sun et al., 2007; Toney et al., 2012; Theroux et al., 2013; Nakamura et al., 2014; Longo et al., 2016) and Group I Isochrysidales have been found so far only in freshwater to oligohaline lakes (0–5 g/kg; Theroux et al., 2010; Crump et al., 2012; D'Andrea et al., 2016; Longo et al., 2016, 2018; Plancq et al., 2018). The mechanism restricting phylotypes to specific salinity ranges is unknown but may be related to different evolutionary transitions of the phylotypes from marine to terrestrial environments (Simon et al., 2013). Environments characterized by large surface salinity and temperature gradients are extremely useful to study the spatial partition of LCA-producing Isochrysidales and the effects of these physical parameters on LCA distributions.

The Baltic Sea is characterized by a horizontal pelagic salinity gradient over 2000 km. In contrast to most estuaries, the Baltic Sea salinity gradient is relatively stable as tidal influences are negligible. The seasonal variation in surface salinity is low (<1 g/kg) and the water residence time is relatively long (ca. 30 years) (Meier and Kauker, 2003; Döös et al., 2004; Meier et al., 2006; Reissmann et al., 2009). Therefore, the Baltic Sea provides an ideal system to investigate the influence of salinity on the distribution patterns of species (Herlemann et al., 2011; Josefson, 2016).

Recently, Kaiser et al. (2017) found three distinct types of LCA distribution (and their mixing types) in surface sediments from the marine Skagerrak and the entire brackish Baltic Sea. Type A distribution, found in the marine Skagerrak and the southern Baltic Sea (8–34 g/kg), resembles the LCA distribution in E. huxleyi with the presence of $C_{38:3a}$ and $C_{38:2}$ ethyl (Et) and methyl (Me) LCAs and the C_{39}Et LCAs. Type B distribution, found in surface sediments from the mesohaline central Baltic Sea (6–8 g/kg), is similar to R. lamellosa and I. galbana LCA distributions, although these haptophytes have never been observed in the Baltic Sea (Hällfors, 2004). It is characterized by the presence of the $C_{38:4}$Et LCA, the absence of $C_{38:3}$Me and $C_{38:2}$Me LCAs, and the replacement of the C_{39}Et by C_{39}Me LCAs. Type C distribution, found in the oligohaline northern Baltic Sea (<6 g/kg), is known from some lake sediments (Zink et al., 2001; Theroux et al., 2010; Toney et al., 2012; Longo et al., 2016). High relative amounts of $C_{37:4}$Me and $C_{38:4}$Et LCAs, the potential presence of the $C_{37:3}$Me LCA isomer and the presence of $C_{38:3}$Me, $C_{38:2}$Me, $C_{39:3}$Et, and $C_{39:3}$Me LCAs characterize this type of distribution. The authors suggested that these different types of LCA distributions are very likely related to different LCA-producing organisms, whose spatial distribution may be related to their salinity tolerance.

The chromatographic method used in Kaiser et al. (2017), however, was not optimized for LCA analysis of sediments from brackish and freshwater systems. The recent establishment of a new method for LCA analysis by gas chromatography (GC) considerably improved the study of LCA producers. Routinely, LCAs are analysed using non-polar capillary column stationary phases composed of poly(methylphenylsiloxane) that cannot separate the full distribution of LCAs. Longo et al. (2013), and more recently Dillon et al. (2016) and Zheng et al. (2017) have shown that mid-polar GC capillary columns coated with poly(trifluoropropylmethylsiloxane) stationary phase provide superior LCA separation by fully resolving partially co-eluting LCAs. This methodological improvement provided separation of previously unresolved C_{37}, C_{38} and C_{39} tri-unsaturated positional LCA isomers with double bound positions at Δ^{14}, Δ^{21}, Δ^{28} (noted "b"; e.g., $C_{37:3b}$) contrasting to the common Δ^{7}, Δ^{14}, Δ^{21} pattern (noted "a"; e.g., $C_{37:3a}$) (Longo et al., 2013; Dillon et al., 2016). These isomers and particularly the $C_{37:3b}$ LCA isomer seem to be produced exclusively by Group I Isochrysidales, representing a biomarker specific for these haptophytes (Longo et al., 2013, 2016, 2018; Dillon et al., 2016).

The main objective of this present study was to test the hypothesis that LCA distribution types A, B and C reflect the LCA distributions of groups III, II and I Isochrysidales, respectively. For this purpose, we re-analysed surface sediments from the Skagerrak and the broader Baltic Sea using the GC method recently published in Zheng et al. (2017). The application of the new GC method did not considerably affect the results and conclusions from Kaiser et al. (2017). Furthermore, we have generated haptophyte DNA sequence data of some surface sediments in an attempt to identify potential LCA source organisms. The results confirm the presence of Group I and II Isochrysidales in the Baltic Sea, solving the eighteen-year-long mystery of the source of LCAs in the Baltic Sea (Schulz et al., 2000; Blanz et al., 2005; Kaiser et al., 2017).

2. Material and methods

2.1. Surface sediments from the Baltic Sea

The Baltic Sea is a shallow, semi-enclosed and intra-continental shelf sea with an average water depth of 55 m (Fig. 1). It can be divided into several main geographical regions such as the northern Baltic Sea (Bothnian Sea and Bothnian Bay), the central Baltic Sea (including the Eastern Gotland Basin and the Landsort Deep), and the southern Baltic Sea (Arkona Basin). The greatest depth in the Baltic Sea (459 m) is reached in the Landsort Deep. The Baltic Sea is further connected to the Kattegat Sea by shallow straits and to the North Sea by the Skagerrak.

The surface salinity distribution is characterized by a strong gradient in the south-western Baltic Sea between the Skagerrak (35 g/kg) and the Arkona Basin (8 g/kg). From the southern to the northernmost Baltic Sea, the surface salinity decreases from 8 to <3 g/kg with values around 6–7 g/kg in the central Baltic Sea. The large freshwater input from rivers drives the large-scale surface circulation in the Baltic Sea. On its way to the North Sea the freshwater mixes with Baltic Sea water creating a brackish surface layer flowing westward. This outflowing water is compensated by a restricted inflow of saline water from the North Sea through the shallow Kattegat Sea. In the different regions of the Baltic Sea, the surface current circulation is anti-clockwise.

The surface sediments (0–1 cm) re-analysed here (Fig. 1; Table 1) are part of a larger dataset representing the complete Baltic Sea. Except for two surface sediments sampled during expedition POS507 on board R/V Poseidon (Arz et al., 2017), this dataset has been published for other purposes in Kaiser and Arz (2016) and Kaiser et al. (2017). For technical reasons, DNA and LCA analyses were not always done on exactly the same sediments. However, the sediments can be considered as relatively homogenous at a basin scale (Kaiser et al., 2017).

2.2. DNA extraction and analysis

DNA was extracted from nine surface sediments (500 mg dry weight) using the FastDNA™ SPIN Kit for Soil (MP Biomedicals, OH, USA) according to the manufacturer's instructions. The DNA extracts were then purified using a DNeasy PowerClean Pro Cleanup Kit (Qiagen, Carlsbad, CA, USA) according to the manufacturer's instructions. Genomic DNAs were amplified by

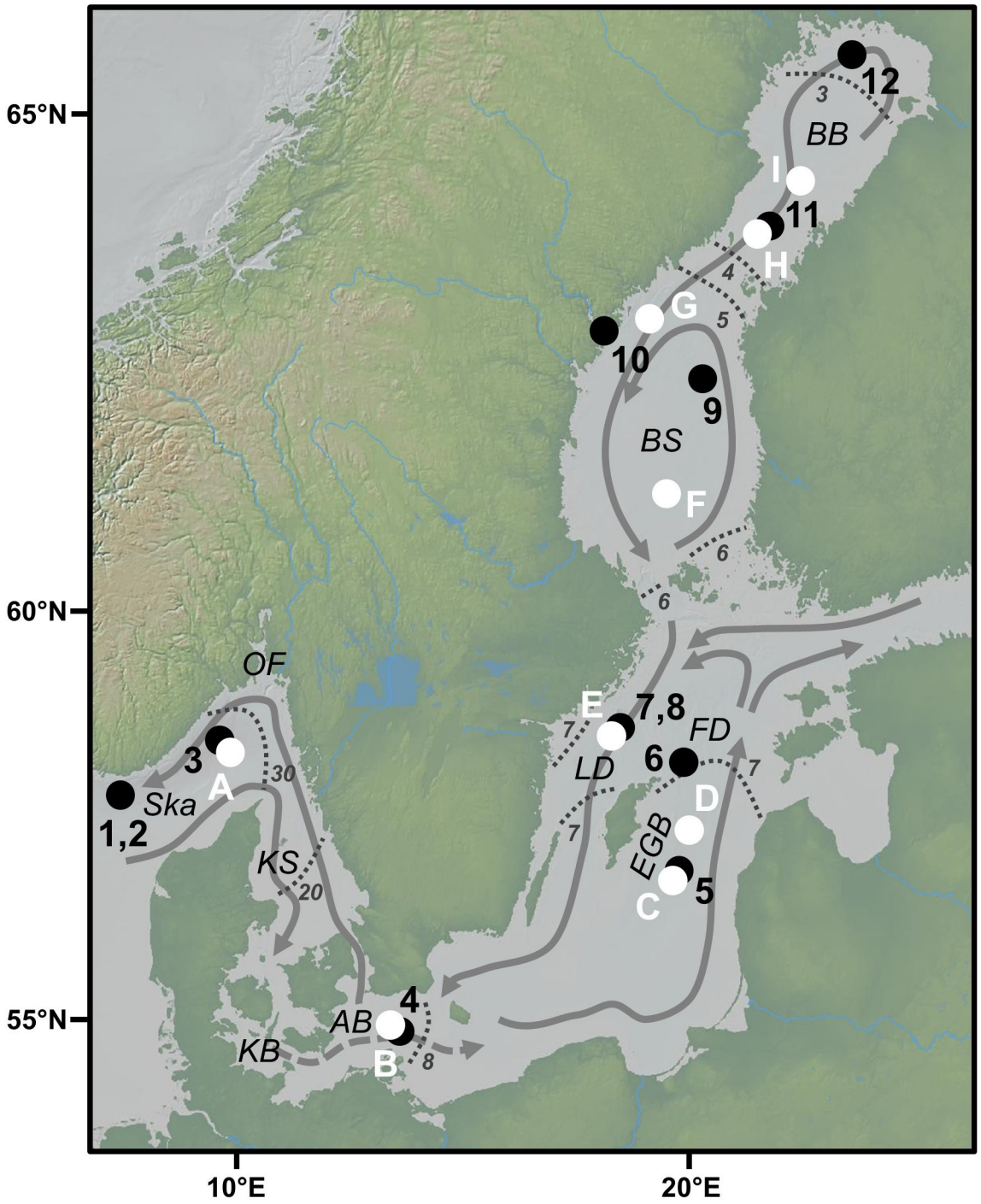

Fig. 1. Sample location and surface isohalines (dotted lines) in the Baltic Sea. 1–12: surface sediments analysed for LCA distributions; A–I: surface sediments analysed for DNA sequencing. The surface circulation is illustrated (grey arrows) based on Meier et al. (2006); the dashed grey arrow represents the pathway of deep waters entering the southern Baltic Sea. Ska: Skagerrak; OF: Oslofjord; KS: Kattegat Sea; KB: Kiel Bight; AB: Arkona Basin; EGB: Eastern Gotland Basin; LD: Landsort Deep; FD: Farö Deep; BS: Bothnian Sea; BB: Bothnian Bay.

haptophyte-specific primers targeting 18S rRNA gene region, Prym-429F and Prym-887R (Coolen et al., 2004). PCR conditions were modified from Coolen et al. (2004) to reduce non-specific amplification: denaturing 4 min at 96 ºC, followed by 35 cycles including denaturing (30 s at 94 ºC), 40 s of primer annealing at 61.5 ºC, and primer extension (40 s at 72 ºC). A final extension was performed at 72 ºC (10 min).

Sedimentary DNA from a Chinese salt lake proven to have Group II Isochrysidales was used as a positive control for PCR. Triplicate reactions of each sample were run, and the PCR products were examined by gel electrophoresis and purified with the Axy-Prep DNA Gel Extraction Kit (Axygen, USA). Cloning was performed using the pGM-T Cloning Kit (Tiangen Biotech, Beijing, China) with TOP10 competent cells according to the manufacturer's instructions. Sanger sequencing of the cloned products was performed on an ABI 3730XL capillary sequencer (Applied Biosystems, Foster City, CA). Shanghai Majorbio Bio-Pharm Technology Co., Ltd., China performed the cloning and sequencing.

Table 1
Sediment locations, environmental data and selected alkenone ratios and percentages obtained in the present study.

ID	ID in Kaiser et al. (2017)	Sediment name	Lat N	Long E	Region	Water depth (m)	SST[a] (°C)	SSS[b] (g/kg)	U^{K}_{37}	U^{K}_{37ab}	$U^{K'}_{37}$	$U^{K'}_{37ab}$	$U^{K'}_{37}$	$U^{K'}_{37ab}$	% $C_{37:4}$	% $C_{37:4'}$	RIK_{37}	RIK_{38E}	$RC_{38}Me/RC_{38}Et$	RC_{37}/RC_{38}
1	5	EMB046/11-2	57.911	7.294	Skagerrak	297	9.8	34.6	0.41	0.41	0.97	0.97	0.43	0.43	1.7	1.7	1.00	1.00	1.16	1.34
2	8	EMB046/15-1	57.635	7.299	Skagerrak	301	10	34.6	0.37	0.37	0.97	0.97	0.40	0.40	1.7	1.7	1.00	1.00	2.23	1.64
3	4	EMB046/18-2	58.412	9.801	Skagerrak	543	9.9	34.5	0.37	0.37	0.97	0.97	0.40	0.40	1.7	1.7	1.00	1.00	2.70	1.69
4	66	M86-1/18-2	54.852	13.433	Arkona Basin	41	9.4	7.6	0.47	0.47	0.92	0.92	0.53	0.53	3.7	3.7	1.00	1.00	0.59	1.28
5	19	EMB1215/3-2	57.083	19.985	Eastern Gotland Basin	189	9	6.9	0.41	0.41	0.94	0.94	0.45	0.45	3.1	3.1	1.00	1.00	0.00	1.26
6	20	EMB1215/8-1	58.102	19.718	Färö Deep	165	8.6	7.1	0.12	0.12	0.84	0.84	0.27	0.27	11.8	11.8	1.00	1.00	0.00	1.62
7	49	M86-1/37-4	58.673	18.518	Landsort Deep	250	8.4	6.8	0.08	0.08	0.83	0.83	0.24	0.24	13.4	13.4	1.00	1.00	0.00	1.84
8	14	EMB1215/9-2	58.603	18.711	Landsort Deep	214	8.5	6.8	0.21	0.21	0.83	0.83	0.37	0.37	11.5	11.5	1.00	1.00	0.00	0.52
9	63	M86-1/43-3	62.416	20.082	Bothnian Sea	89	6.7	5.2	0.12	0.12	0.77	0.78	0.35	0.33	16.6	15.8	0.91	0.75	0.41	1.10
10	26	P435/8-5	62.778	18.049	Bothnian Sea	91	5.8	5	0.10	0.09	0.79	0.80	0.30	0.28	15.8	14.9	0.91	0.84	0.44	2.39
11	55	M86-1/48-2	63.833	21.583	Bothnian Bay	59	5.9	3.1	-0.24	-0.22	0.53	0.59	0.25	0.21	39.5	35.1	0.78	0.75	0.44	1.55
12	44	M86-1/45-4	65.445	23.298	Bothnian Bay	79	6.2	2.8	-0.16	-0.14	0.61	0.68	0.24	0.19	32.8	27.8	0.74	0.85	0.33	0.87

[a] Sea surface temperature.
[b] Sea surface salinity. See Fig. 1 for sediment locations.

A bioinformatics pipeline was used to generate base-calls and associated quality scores, remove vector sequences and assemble forward and reverse reads into full-length sequences for each of the cloned PCR amplicons (Ewing and Green, 1998). Only sequences greater than 400 bp and with a complete forward and reverse primer were retained. Sequences have been deposited in GenBank under accession numbers MH472563 to MH472566. Isochrysidales sequences were identified through BLAST searches against the NCBI database, and Chimera checking was performed using ChimeraSlayer in QIIME/1.9.1.

The raw capillary sequence data yielded 146 Isochrysidales sequences after editing. Operational Taxonomic Units (OTUs) were clustered using UCLUST with a 97% cut-off criterion. Representative sequences were picked for each OTU with the ''pick_rep_set.py" command in QIIME/1.9.1. Representative sequences were then aligned by PyNast (Caporaso et al., 2010) against the aligned haptophyte database in Gran-Stadniczeñko et al. (2017). The new sequences were aligned with Prymnesium faveolatum and phylogenies were inferred using the Randomized Axelerated Maximum (RAxML) program (Huelsenbeck and Crandall, 1997; Stamatakis, 2014) to reconstruct a maximum likelihood tree. Rapid bootstrapping with 1000 bootstrap replicates and an ML search under the GAMMA model of rate heterogeneity were conducted.

2.3. LCA extraction and analysis

Surface sediments (n = 15) were extracted and fractionated as published in Kaiser et al. (2017). The alkenoate-free fractions containing LCAs were re-analysed by gas chromatography using a Rtx-200 column (105 m × 250 mm i.d. × 0.25 mm film thickness) as described in Zheng et al. (2017). This capillary column provides the best separation of LCAs and baseline resolution of close-eluting LCAs including double bond positional isomers. After splitless injection, the initial temperature was set at 50 °C (held 2 min), followed by a first ramp of 20 °C/min to 255 °C and a second ramp of 3 °C/min to 320 °C (held 25 min).

The following ratios of isomeric ketones (RIK) were used to examine the species specific and environmental significance of the isomers, where 'a' is the common $\Delta^{7,14,21}$ isomer and 'b' is the novel $\Delta^{14,21,28}$ isomer (Longo et al., 2016):

$$RIK_{37} = [C_{37:3a}Me] / ([C_{37:3a}Me] + [C_{37:3b}Me]) \quad (1)$$

$$RIK_{38E} = [C_{38:3a}Et] / ([C_{38:3a}Et] + [C_{38:3b}Et]) \quad (2)$$

The following indices (D'Andrea et al., 2011; Longo et al., 2016; Zheng et al., 2016) were used in order to compare the possible differences related to the GC methods used in Kaiser et al. (2017) and in the present study:

$$U^{K}_{37} = U^{K}_{37ab} = [C_{37:2}Me] - [C_{37:4}Me] / ([C_{37:2}Me] + [C_{37:3a}Me] + [C_{37:3b}Me] + [C_{37:4}Me]) \quad (3)$$

$$U^{K}_{37a} = [C_{37:2}Me] - [C_{37:4}Me] / ([C_{37:2}Me] + [C_{37:3a}Me] + [C_{37:4}Me]) \quad (4)$$

$$U^{K'}_{37} = U^{K'}_{37ab} = [C_{37:2}Me] / ([C_{37:2}Me] + [C_{37:3a}Me] + [C_{37:3b}Me]) \quad (5)$$

$$U^{K'}_{37a} = [C_{37:2}Me] / ([C_{37:2}Me] + [C_{37:3a}Me]) \quad (6)$$

$$U^{K''}_{37} = U^{K''}_{37ab} = [C_{37:3a}Me] / ([C_{37:3a}Me] + [C_{37:3b}Me] + [C_{37:4}Me]) \quad (7)$$

$$K^{w}_{37ab} = [C_{37:3a}Me] / ([C_{37:3a}Me] + [C_{37:3a}Me] + [C_{37:4}Me]) \quad (8)$$

We have also defined here two alternate forms taking into account the LCA isomers to calculate the percentage of $C_{37:4}Me$

relative to $C_{37:3}$Me and $C_{37:2}$Me LCAs as originally established in Rosell-Melé (1998):

$$\%C_{37:4} = \%C'_{37:4} = [C_{37:4}Me]/([C_{37:4}Me] + [C_{37:2}Me] + [C_{37:3a}Me] + [C_{37:3b}Me] + [C_{37:4}Me]) \times 100 \quad (9)$$

$$\%C'_{37:4} = [C_{37:4}Me]/([C_{37:4}Me] + [C_{37:2}Me] + [C_{37:3a}Me] + [C_{37:4}Me]) \times 100 \quad (10)$$

3. Results

3.1. LCA distributions in Baltic Sea surface sediments

The Zheng et al. (2017) GC method for LCA analysis improved the peak resolution considerably compared to the GC method used in Kaiser et al. (2017) (Fig. 2). More specifically, the new method revealed the presence of three LCA isomers ($C_{37:3b}$Me, $C_{38:3b}$Et and $C_{38:3b}$Me) in the sediments from the Bothnian Sea and the Bothnian Bay. The North Sea sediment (used only here as reference for the open ocean) and Skagerrak sediments (sediments 1–3; Table 2) had a distribution containing $C_{37:4}$Me, $C_{37:3a}$Me, $C_{37:2}$Me, $C_{38:3a}$Et, $C_{38:2}$Et, $C_{38:3a}$Me, $C_{38:2}$Me, $C_{39:3a}$Et and $C_{39:2}$Et LCAs. In the Arkona Basin sediment (sediment 4; Table 2), the distribution was very similar, but the relative content of $C_{38:3}$Et LCA was slightly higher. The sediments of the Eastern Gotland Basin and the Landsort Deep (sediments 5–8; Table 2) were characterized by the absence of $C_{38:3a}$Me, $C_{38:2}$Me, $C_{39:3a}$Et, and $C_{39:2}$Et LCAs, and the presence of $C_{38:4}$Et, $C_{39:3a}$Me and $C_{39:2}$Me LCAs. The distributions of both Bothnian Sea and Bothnian Bay sediments (sediments 9–12; Table 2) showed the presence of $C_{37:4}$Me, $C_{37:3a}$Me, $C_{37:2}$Me, $C_{38:3a}$Et, $C_{38:2}$Et, $C_{38:3a}$Me, $C_{38:2}$Me, $C_{39:3a}$Et and $C_{39:2}$Et LCAs. The relative content of both $C_{37:4}$Me and $C_{38:4}$Et LCAs were particularly high (%$C_{37:4}$ was 14–41) and LCA isomers were present exclusively in those sediments. The different LCA distributions dependent on the various Baltic Sea regions is illustrated in Fig. 3 using the $RC_{38}Me/RC_{38}Et$ ratio (Table 1).

3.2. DNA sequencing and phylogenetic reconstruction

Genomic DNAs were successfully extracted from all sediments, and they all yielded positive amplification for haptophytes. However, the number of clones recovered for DNA sequencing differed from sediment to sediment. In total 146 Isochrysidales sequences were recovered from 264 sequenced clones, including 50 out of 50 for sediment H (Bothnian Sea), 20 out of 26 for sediment I (Bothnian Bay), 16 out of 28 for sediment F (Bothnian Sea), 13 out of 40 for sediment B (Arkona Basin), 13 out of 23 for sediment G (Bothnian Sea), 13 out of 23 for sediment C (Eastern Gotland Basin), 10 out of 25 for sediment E (Landsort Deep), 9 out of 18 for sediment D (Landsort Deep), and 2 out of 4 for sediment A (Skagerrak) (Table 3). Four Isochrysidales OTUs were identified

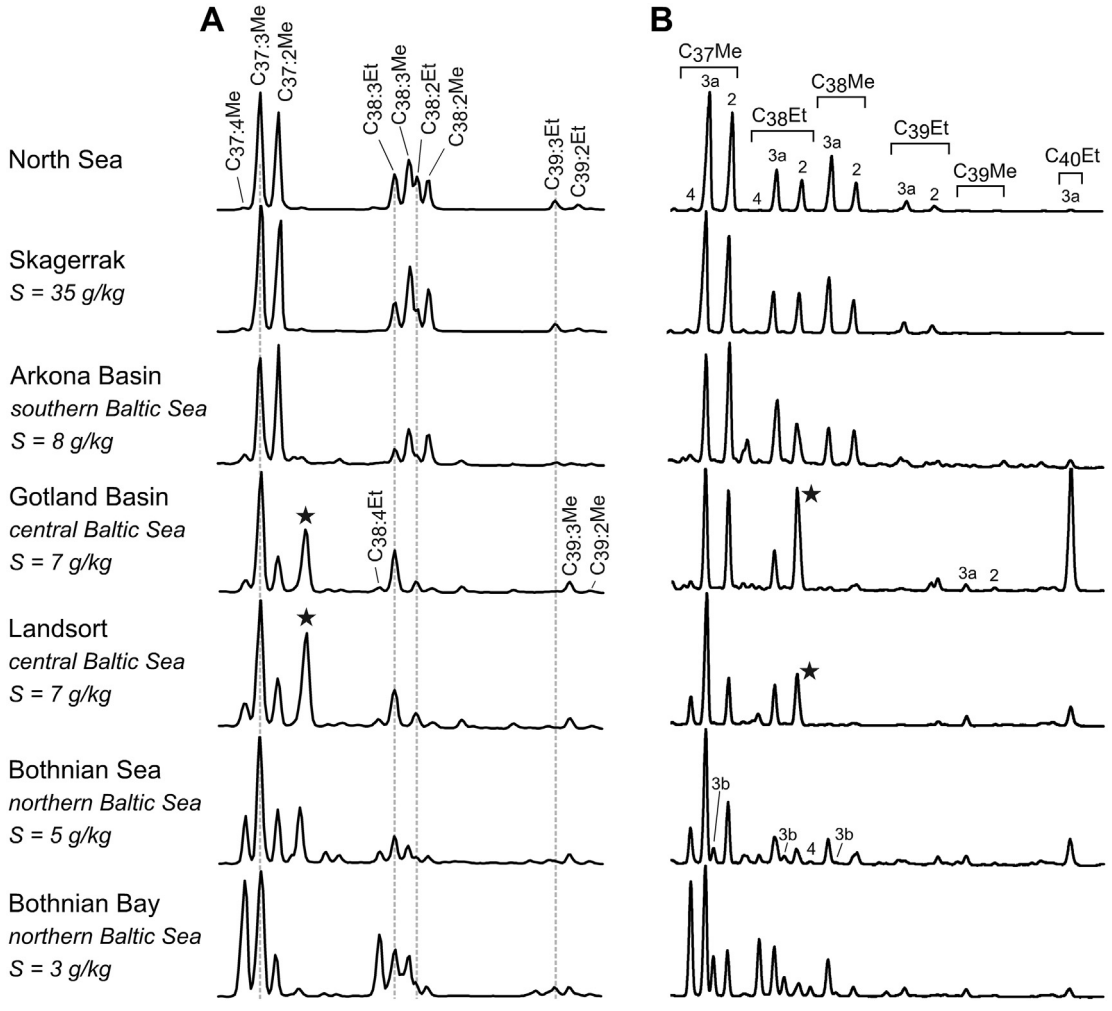

Fig. 2. GC-FID chromatograms of sediments analysed with: (A) Kaiser et al. (2017) and (B) Zheng et al. (2017) methods. Note that the $C_{38:2}$Et LCA is potentially co-eluting with a C_{32} hopanoic acid methyl ester (black star), which is present in the ketone fraction of the surface sediments from the Eastern Gotland Basin and the Landsort Deep (Kaiser et al., 2017).

Table 2
Abundances of major alkenones (ng/g dry wt sediment) in each of the sediments studied.

ID	ID in Kaiser et al. (2017)	$C_{37:4}$Me (ng/g)	$C_{37:3a}$Me (ng/g)	$C_{37:3b}$Me (ng/g)	$C_{37:2}$Me (ng/g)	$C_{38:4}$Et (ng/g)	$C_{38:3a}$Et (ng/g)	$C_{38:3b}$Et (ng/g)	$C_{38:2}$Et (ng/g)	$C_{38:4}$Me (ng/g)	$C_{38:3a}$Me (ng/g)	$C_{38:3b}$Me (ng/g)	$C_{38:2}$Me (ng/g)	$C_{39:4}$Et (ng/g)	$C_{39:3a}$Et (ng/g)	$C_{39:3b}$Et (ng/g)	$C_{39:2}$Et (ng/g)	$C_{39:3}$Me (ng/g)	$C_{39:2}$Me (ng/g)	$C_{40:3}$Et (ng/g)
1	5	87	2938	n.d.[a]	2249	n.d.	905	n.d.	913	n.d.	1352	n.d.	759	n.d.	245	n.d.	197	14	20	40
2	8	114	3844	n.d.	2548	n.d.	838	n.d.	388	n.d.	1863	n.d.	870	n.d.	225	n.d.	81	32	22	24
3	4	131	4538	n.d.	2976	n.d.	847	n.d.	377	n.d.	2223	n.d.	1076	n.d.	245	n.d.	94	29	25	23
4	66	51	616	n.d.	693	13	475	n.d.	329	n.d.	245	n.d.	238	n.d.	51	n.d.	35	12	46	60
5	19	276	4654	n.d.	3846	89	1624	n.d.	5257	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	263	122	6511
6	20	1564	8480	n.d.	3182	459	2623	n.d.	5065	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	677	108	3684
7	49	3233	15,801	n.d.	5081	1416	4723	n.d.	6983	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1219	172	2913
8	14	397	1913	n.d.	1135	77	568	n.d.	6006	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	134	29	2221
9	63	21	70	7	37	9	48	16	21	n.d.	28	n.d.	10	n.d.	12	n.d.	10	5	n.d.	61
10	26	141	522	54	229	33	142	27	74	9	109	n.d.	n.d.	n.d.	22	n.d.	39	43	11	149
11	55	1187	1362	380	457	628	549	184	166	92	425	36	111	57	120	17	18	99	24	123
12	44	263	407	144	132	119	541	94	63	20	190	7	51	8	37	18	n.d.	24	n.d.	66

[a] Not detected. For the location of each sediment see Table 1 and Fig. 1.

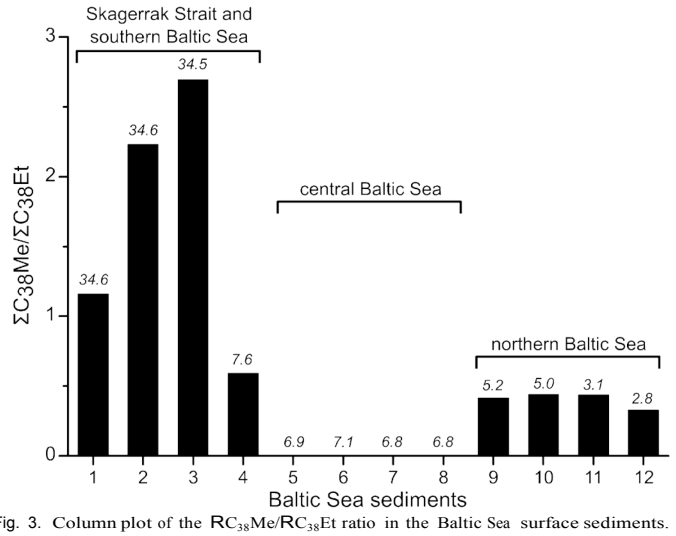

Fig. 3. Column plot of the $RC_{38}Me/RC_{38}Et$ ratio in the Baltic Sea surface sediments. Numbers refer to sediments listed in Table 1. Surface salinity values (g/kg) at the sampling sites are specified in italics above each column.

from the sediments, and we refer to them as Baltic OTU1, 2, 3, and 4 (Fig. 4).

The Isochrysidales representative sequences fell into two of the three known groups of LCA producers. Baltic OTU1 fell into Group II Isochrysidales and clustered with sequences from Lake Bonney, Antarctica (GU969080 and GU969079) (Bielewicz et al., 2011). The other three OTUs all branched within the Group I Isochrysidales, Baltic OTU2 and OTU3 shared most recent common ancestry with each other and clustered with but separately from other Group I Isochrysidales, while Baltic OTU4 was identical to the uncultured haptophyte clone OTU5 from BrayaSø, Greenland (HQ446255). Our cloning and sequencing efforts failed to detect Group III Isochrysidales representatives despite clear LCA signatures and the fact that the Coolen et al. (2004) primers should have amplified this group. In terms of spatial distributions, Baltic OTU1 was found in all sediments of the study area. Baltic OTU4 was found in the complete Baltic Sea (sediments I, H, G, E, C and B), and was the most widely occurring Group I Isochrysidales. It was particularly abundant in the Bothnian Bay. Baltic OTU2 was only found in the Arkona Basin (sediment B) and Baltic OTU3 was only found in the Landsort Deep (sediment D). Those two OTUs were less abundant and distinct from other Group I Isochrysidales.

4. Discussion

4.1. Baltic Sea LCA producers: evidence from LCA distributions

The LCA distributions of the Baltic Sea surface sediments (Fig. 2) suggest the presence of all three main groups of LCA-producing Isochrysidales (Kaiser et al., 2017). Similar to the reference sediment from the North Sea, the Skagerrak sediment has a distribution (type A distribution in Kaiser et al., 2017) characteristic for the ''marine" Group III Isochrysidales (Theroux et al., 2010; Longo et al., 2016). The Arkona Basin distribution is very similar, but has a slightly higher relative content of $C_{38:3a}$Et LCA possibly indicating the presence of ''brackish" Group II Isochrysidales as well. The sediments of the Eastern Gotland Basin and the Landsort Deep have LCA distributions typical for Group II Isochrysidales (type B distribution in Kaiser et al., 2017) characterized by the absence of $C_{38:3a}$Me, $C_{38:2}$Me, $C_{39:3a}$Et, and $C_{39:2}$Et LCAs, and the presence of $C_{39:3a}$Me, $C_{39:2}$Me and $C_{40:3a}$Et LCAs (Longo et al., 2016). The presence of $C_{39:3a}$Me LCA and tri-unsaturated isomers in the distribution of both Bothnian Sea and Bothnian Bay sediments suggests a

Table 3
Number of amplified Isochrysidales partial 18S rDNA gene sequences and phylogenetic assignment. The frequency of the presence of Baltic OTUs in the different surface sediment is indicated.

Lat N	Long E	Region	Water depth (m)	Surface salinity (g/kg)	No. of Isochrysidales sequences relative to all haptophyte sequences	No. of Group I Isochrysidales sequences	No. of Group II Isochrysidales sequences	Baltic OTU1	Baltic OTU2	Baltic OTU3	Baltic OTU4
58.429	9.477	Skagerrak	509	34.5	2/4	0	2	2			
54.852	13.433	Arkona Basin	41	7.6	13/40	3	10	10	2		1
57.083	19.985	Eastern Gotland Basin	189	6.9	13/23	5	8	8			5
57.267	19.796	Landsort Deep	207	6.9	9/18	3	6	6		3	
58.641	18.265	Landsort Deep	444	6.8	10/25	3	7	7			3
61.085	19.579	Bothnian Sea	127	5.2	16/28	0	16	16			
62.845	18.889	Bothnian Sea	206	5.0	13/23	6	7	7			6
63.833	21.583	Bothnian Bay	59	3.1	50/50	39	11	11			39
64.204	22.029	Bothnian Bay	109	3.0	20/26	15	5	5			15

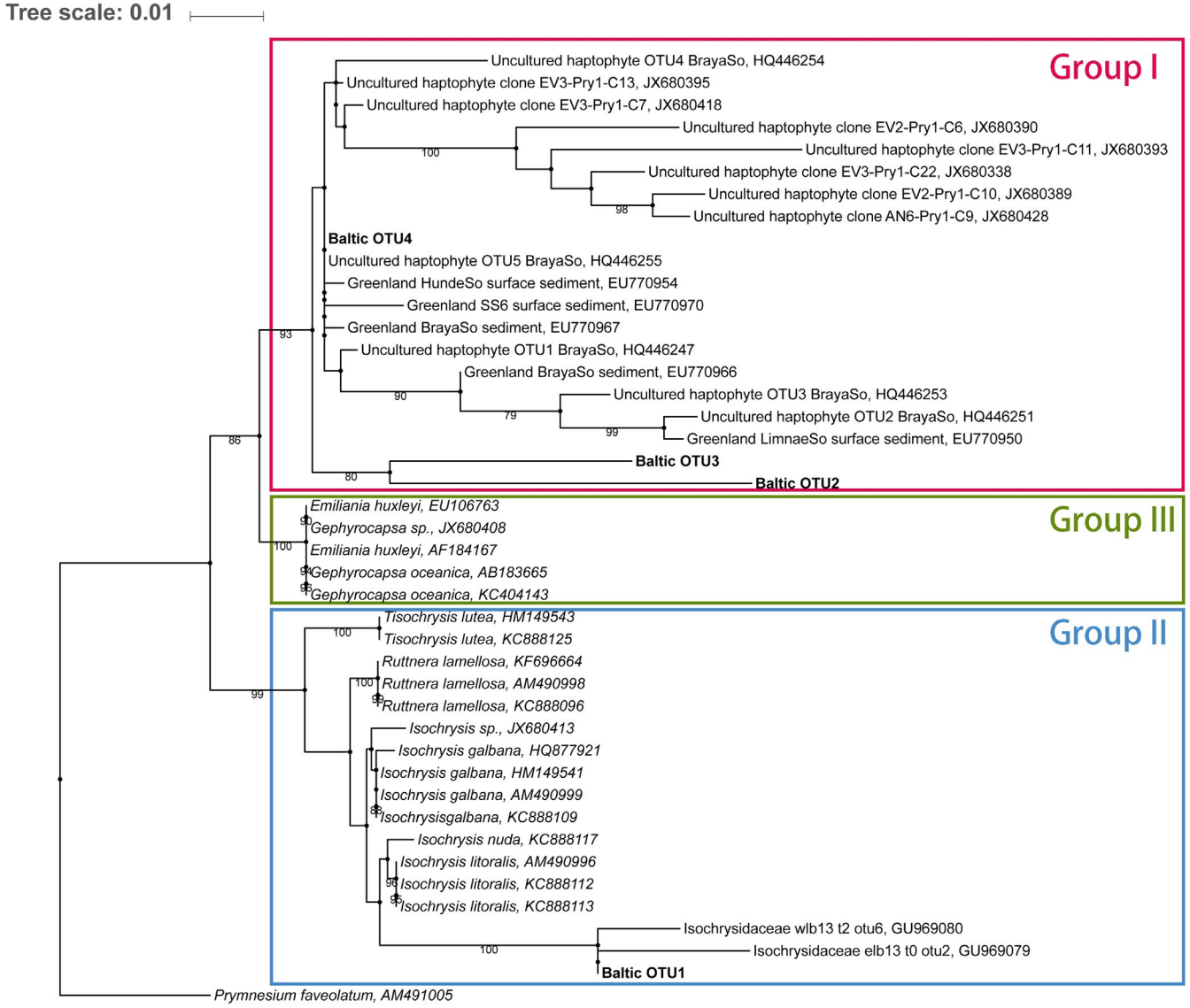

Fig. 4. Maximum likelihood phylogenetic tree of haptophyte partial 18S rRNA genes. Baltic OTU1, 2, 3 and 4 represent the four Isochrysidales OTUs identified from the Skagerrak and Baltic Sea surface sediments. The scale bar represents evolutionary distance for the number of changes per site.

mixture of Group II and ‘‘freshwater” Group I Isochrysidales (type C distribution in Kaiser et al., 2017).

Longo et al. (2016) suggests using $C_{37:3b}$Me, $C_{37:4}$Me, and the sum of $C_{37:2}$Me and $C_{37:3a}$Me LCAs as end-members for, respectively, Group I, II and III distributions as a broad tool to estimate the presence of Group I, II and III Isochrysidales in an environment. This approach allows differentiating the surface sediments from the southern, central and northern Baltic Sea (Fig. 5A). The results

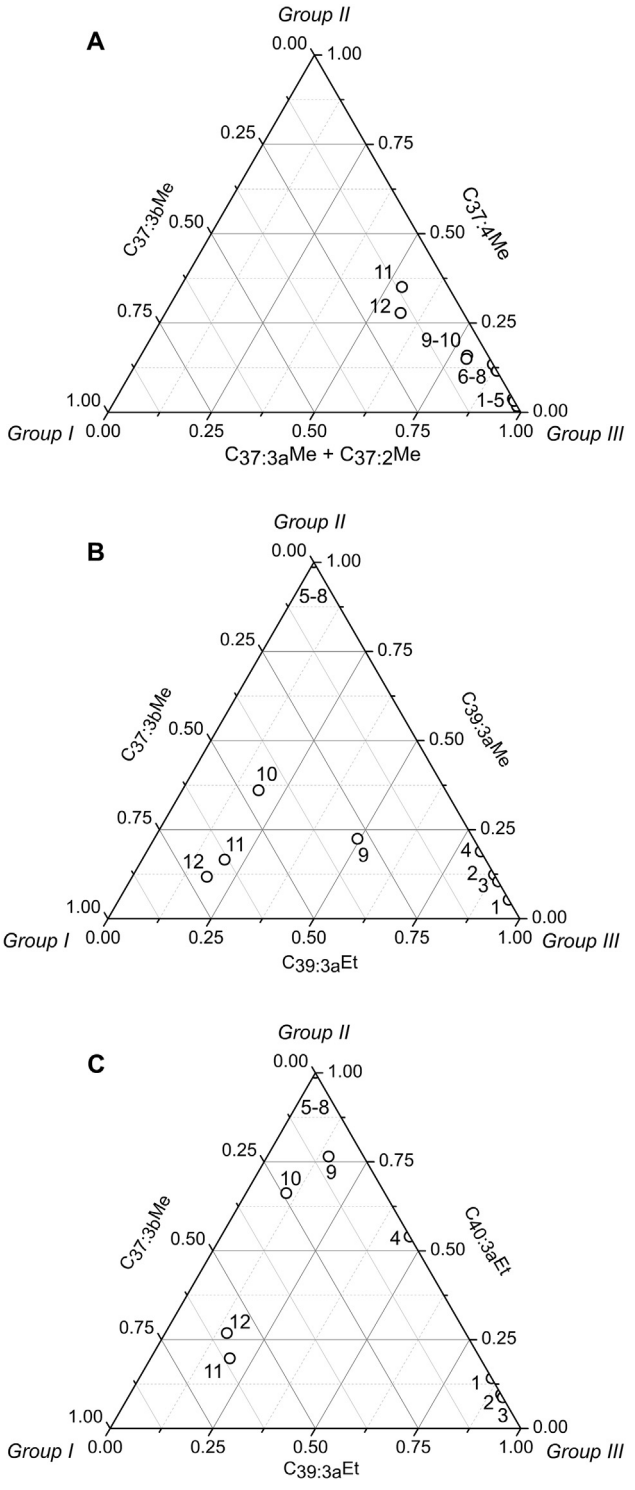

Fig. 5. Ternary diagrams based on the proportion of specific LCAs in the surface sediments: (A) $C_{37:4}$Me, $C_{37:3b}$Me, and the sum of $C_{37:3a}$Me and $C_{37:2}$Me; (B) $C_{39:3a}$Et, $C_{39:3a}$Me, and $C_{37:3b}$Me; (C) $C_{39:3a}$Et, $C_{40:3a}$Et, and $C_{37:3b}$Me. Group I, II and III Isochrysidales end-members are shown. Numbers refer to sediments listed in Table 2.

suggest the presence of Group II and III Isochrysidales in the mixoeuhaline Skagerrak and the mesohaline southern and central Baltic Sea (sediments 1–8), and a mixture of Group I and II Isochrysidales in the oligohaline northern Baltic Sea (sediments 9–12).

However, as $C_{37:4}$Me LCA and the sum of $C_{37:2}$Me and $C_{37:3a}$Me LCAs are not specific for, respectively, Group II and III Isochrysidales, we suggest using instead $C_{39:3a}$Me or $C_{40:3a}$Et LCA to estimate the presence of Group II Isochrysidales, and $C_{39:3a}$Et LCA as an indicator for Group III Isochrysidales (Fig. 5B and C).

Considering that $C_{37:3b}$Me, $C_{40:3a}$Et and $C_{39:3a}$Et LCAs provide the clearest grouping of the sediments and suggests LCA distribution characteristics for Group II and III Isochrysidales in the Skagerrak (sediments 1–3), for mainly Group II Isochrysidales in the central Baltic Sea (sediments 5–8) and for a mixture of Group I and II Isochrysidales in the Bothnian Bay (sediments 11 and 12). As expected, the Arkona Basin sediment (sediment 4) occurs between the Skagerrak and the central Baltic Sea sediments, and the Bothnian Sea sediments (sediments 9 and 10) between the central Baltic Sea and the Bothnian Bay sediments. As $C_{39:3a}$Et LCA is also present in the LCA distribution of Group III Isochrysidales (Zheng et al., 2017), the results suggest the occurrence of these haptophytes in the northern Baltic Sea. However, considering their assumed salinity tolerance, Group I and III Isochrysidales are very unlikely to mix (Longo et al., 2016). Therefore, the $C_{39:3a}$Et LCA present in the northern Baltic Sea sediments is produced by Group I Isochrysidales.

The RIK_{37} and RIK_{38E} ratios can also be used as a multiple species indicator (Longo et al., 2016). While RIK_{37} values of 1.0 are typical for Group II and III Isochrysidales, values below 1 suggest a mixture of Group I and II Isochrysidales (Longo et al., 2016). Lake sediments strongly suggest that the C_{37} $D^{14,21,28}$ tri-unsaturated LCA isomers are specific biomarkers for Group I Isochrysidales (Theroux et al., 2013; Longo et al., 2013, 2016; D'Andrea et al., 2016; Zheng et al., 2017). In the Baltic Sea surface sediments, RIK_{37} values are around 1 when the surface salinity is >6 g/kg (Skagerrak, southern and central Baltic Sea) and they drop to values between 0.74 and 0.92 when the salinity is below 6 g/kg (Bothnian Sea and Bothnian Bay) due to the presence of the $C_{37:3b}$Me LCA isomer (Fig. 6A).

Since Group II Isochrysidales apparently do not biosynthesiz C_{38}Me LCAs in contrast to Group I Isochrysidales, the RC_{38}Me/RC_{38}Et ratio can help distinguish between Group I and Group II Isochrysidales. Indeed, RC_{38}Me/RC_{38}Et values are 0.0 in the central Baltic Sea and ca. 0.3–0.4 in the northern Baltic Sea (Table 1; Fig. 3). This approach can be applied in environments where only Group I and Group II Isochrysidales occur as Group III Isochrysidales produce C_{38}Me LCAs as well. However, as mentioned earlier, Group I and III Isochrysidales are very unlikely to mix considering their salinity tolerances (Longo et al., 2016).

4.2. Baltic Sea LCA producers: evidence from DNA sequencing

In order to identify relationships between LCA distributions and the underlying haptophytes responsible, we combined our LCA analysis with molecular phylogenetic tree reconstruction. Baltic OTU picking of the aligned environmental 18S rRNA gene sequence data identified four Baltic OTUs. Three Baltic OTUs associated with Group I LCA-producing Isochrysidales were identified in the entire Baltic Sea, but not the Skagerrak. However, Egge et al. (2014) found Group I Isochrysidales in the surface water of the outer part of the Oslofjord (Fig. 1). This DNA-based evidence contrasts with the LCA distributions suggesting that Group I Isochrysidales are present only in the northern Baltic Sea. Baltic OTU1 belonging to Group II Isochrysidales was found in all sediments, including the Skagerrak. This is in agreement not only with the LCA distributions, but also with the finding of Group II OTUs in the outer Oslofjord (Egge et al., 2014, 2015).

While the LCA distribution in the Skagerrak sediment is typical for Group III Isochrysidales, no DNA sequences from this group were recovered from this sediment. This was unexpected as E. huxleyi has been observed in the Skagerrak water column (Edler et al., 1984; van der Wal et al., 1995; Hällfors, 2004; Blanz et al., 2005)

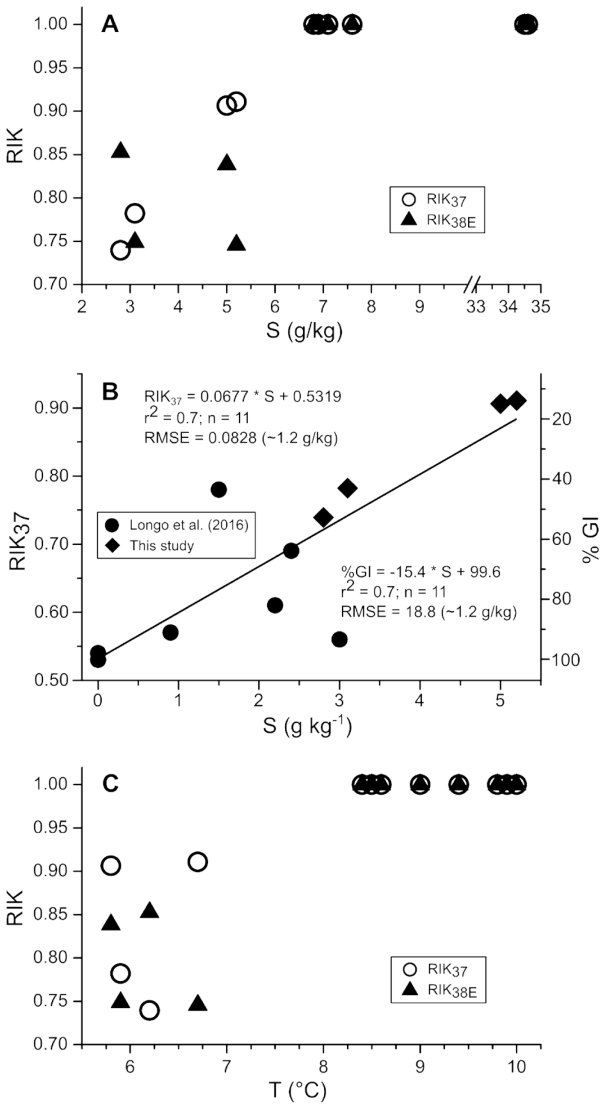

Fig. 6. Cross plots of: (A) surface salinity (S) and RIK_{37} and RIK_{38E} indexes, (B) surface salinity (S) and the RIK_{37} index (left axis) and its equivalent in terms of amount of Group I (GI) Isochrysidales relative to Group II Isochrysidales (% GI; right axis), (C) surface temperature (T) and the RIK_{37} and RIK_{38E} indexes from the surface sediments.

and related environmental DNA sequences have also been detected there (Egge et al., 2014, 2015; Gran-Stadniczeñko et al., 2017). Its absence may be due to the primers we used. While Egge et al. (2014, 2015) could successfully amplify Group III Isochrysidales DNA in the surface waters of the Oslofjord, they did so using a primer targeting Haptophyta V4 SSU rRNA genes. We used different primers developed by Coolen et al. (2004), but failed to detect Group III sequences. The reasons for this are unknown but may be related to sub-optimized DNA amplification conditions, shallow sequencing depth (e.g., only two LCA-producing haptophyte sequences were recovered; Table 3), or PCR inhibitors in this specific sediment.

The discrepancies between the attribution of LCA-producing haptophyte groups based on LCA distribution and Isochrysidales DNA sequences in Baltic Sea sediments may have various explanations. The presence of Group I Isochrysidales OTUs in sediments from the entire Baltic Sea is not reflected in the LCA distributions, which suggests that Group I Isochrysidales are restricted to the Bothnian Bay and the Bothnian Sea (Figs. 2 and 4). Considering that Group I Isochrysidales are thought to be restricted to oligohaline waters (Edvardsen et al., 2016; Longo et al., 2016, 2018), the presence of Group I Isochrysidales DNA in sediments of the entire Baltic Sea may result from the transportation/advection of DNA within surface currents from the northern Baltic Sea at least as far as the Arkona Basin.

Group I Isochrysidales DNA may also be transported to the Skagerrak, as it has been detected in the surface waters of the outer Oslofjord (Fig. 1; Egge et al., 2014), although the fresher, inner part of the fjord may also be a source of Group I Isochrysidales DNA. As environmental DNA may be transported bound to clay minerals or humic acids (Haile, 2009), as can be the case for lipids (Gaines et al., 2009), LCAs produced by Group I Isochrysidales may have also been transported to the southern Baltic Sea, but in quantities too low to be detected. Based on the present results we cannot, however, completely rule out that Group I Isochrysidales are thriving in the entire Baltic Sea and the Skagerrak.

The presence of Group II Isochrysidales DNA in all sediments is in agreement with the LCA distributions as $C_{39:3a}$Me and $C_{40:3a}$Et LCAs seem to be relatively specific to species from Group II Isochrysidales (such as I. galbana and R. lamellosa; Rontani et al., 2004; Sun et al., 2007; Ono et al., 2012) are also present in every sediment (Figs. 2 and 4). This is to be expected as Group II Isochrysidales thrive in waters with a salinity >0.5 g/kg (Longo et al., 2016). Interestingly, the LCA distribution typical for Group II Isochrysidales is found in the sediments from the central Baltic Sea (Eastern Gotland Basin and Landsort Deep). This suggests that in the Baltic Sea, Group II Isochrysidales mainly occupy waters where the salinity is in the range 5–10 g/kg, i.e. where neither Group III nor Group I Isochrysidales can thrive due to the limits of their salinity tolerance. Group II Isochrysidales may also have been partly advected from the northern to the central Baltic Sea.

While our DNA sequencing failed to confirm the presence of Group III Isochrysidales in the Skagerrak sediment, the typical LCA distribution reflects the presence of Group III Isochrysidales in agreement with observations (Edler et al., 1984; van der Wal et al., 1995; Hällfors, 2004; Blanz et al., 2005) and environmental sequencing studies (Egge et al., 2014, 2015; Gran-Stadniczeñko et al., 2017). While E. huxleyi has been observed in the southernmost Baltic Sea (Kiel Bight; Fig. 1; Wasmund et al., 2008; Meier et al., 2014), it has not been observed in the Arkona Basin (Hällfors, 2004). This contradicts the LCA distribution in the Arkona Basin sediment. Therefore, we hypothesize that LCAs produced by Group III Isochrysidales are advected most likely at depth within eastward flowing North Sea water intrusions (Fig. 1).

4.3. Environmental effects on LCA distributions and indexes

The RIK_{37} and RIK_{38E} values in the Bothnian Sea and Bothnian Bay (Fig. 6A) are characteristic for oligohaline (0.5–5 g/kg) environments as evidenced by cultures, lake surface sediments, and suspended particulate matter (Longo et al., 2016, 2018). While it is difficult to tease apart direct effects of salinity vs phylogeny on the relative abundance of $C_{37:3b}$Me LCA (Longo et al., 2016), RIK_{37} values and surface salinity seem to be linearly related in the Baltic Sea which may reflect an increasing amount of Group I relative to Group II Isochrysidales with decreasing salinity, and/or a direct effect of salinity on the relative abundance of $C_{37:3b}$Me LCA.

In order to test these two possibilities, we have added lake surface sediments with RIK_{37} values <1 as published in Longo et al. (2016), and where both Group I and II Isochrysidales were identified. A significant correlation between RIK_{37} and surface salinity was found (Fig. 6B). Lake sediments with Group I Isochrysidales only represent the low salinity (freshwater) end-member with RIK_{37} values of ca. 0.53–0.60 (Longo et al., 2018). Thus, decreasing

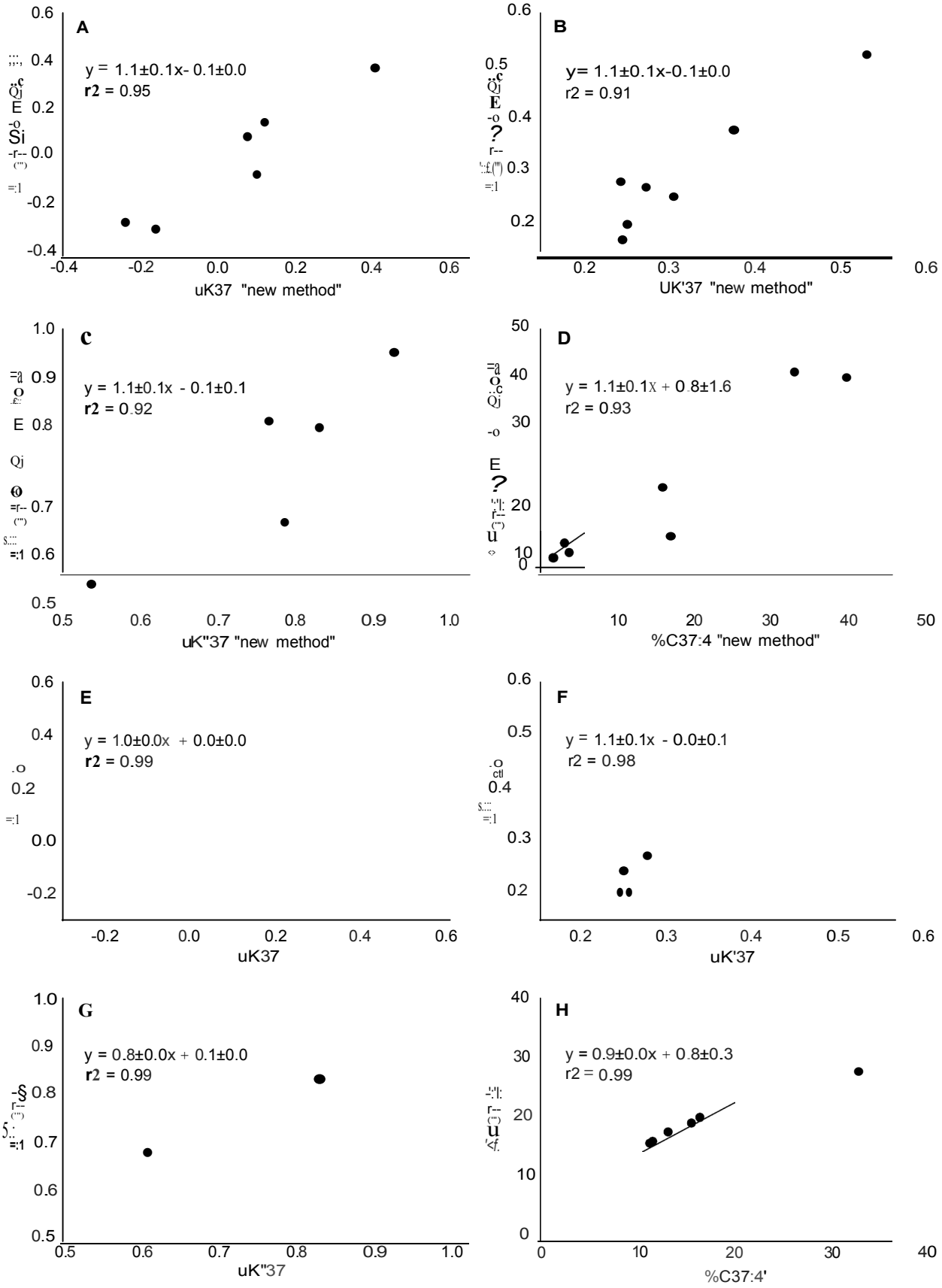

Fig. 7. Comparison of indexes from the same LCA-containing extracts obtained with the "old" (Kaiser et al., 2017) and "new" (Zheng et al., 2017) GC-FID methods: (A) $U^{K'}_{37}$, (B) $U^{K'}_{37}$, (C) $U^{K''}_{37}$ and (D) $\%C_{37:4}$, and for indexes including the isomers (ab) or not (E–H).

RIK_{37} values with decreasing salinity are very likely reflecting the increasing amount of Group I Isochrysidales relative to Group II Isochrysidales. In turn, in an oligohaline environment where only Group I and Group II Isochrysidales are present, RIK_{37} may represent a proxy to estimate surface salinity changes. Based on the present dataset, a surface salinity (S) calibration could be established:

$$S = 0.5319 \times RIK_{37} - 0.0677 \quad (r^2 = 0.7; n = 11; RMSE = 1.2) \quad (11)$$

Following the Longo et al. (2018) concept to quantify the mixing of Group I and Group II Isochrysidales using the RIK_{37} index, RIK_{37} values were converted to amounts of Group I Isochrysidales relative to Group II Isochrysidales (%GI) using RIK_{37} values of 0.53 and 1 as end-members for Group I and Group II Isochrysidales, respectively. Between 0 and ca. 5 g/kg, the relative amount of Group I Isochrysidales is likely varying between 100 and ca. 15%, respectively. By plotting %GI against surface salinity, the following linear relationship was obtained:

$$S = 0.996 \times \%GI - 15.4 \quad (r^2 = 0.7; n = 11; RMSE = 1.2) \quad (12)$$

We have further considered a potential relationship between RIK_{38E} and surface temperature as found in lakes (Longo et al., 2016). Our data suggest no relationship between RIK_{38E} and surface temperature in the Baltic Sea (Fig. 6C), very likely because of the presence of both Group I and Group II Isochrysidales.

Although the full dataset published in Kaiser et al. (2017) was not re-analysed, U^{K}_{37}, $U^{K'}_{37}$, $U^{K''}_{37}$, and $\%C_{37:4}$ values obtained in the present study with a new GC method are very similar to the values obtained in Kaiser et al. (2017) (Fig. 7A–D). Considering the standard errors, the slopes and intercepts are always close to 1 and 0, respectively. Similar results are obtained when comparing indexes which include or exclude the C_{37}Me LCA isomers (Fig. 7E and F). Therefore, the main conclusions on the relationships between these indexes and temperature and/or salinity in Kaiser et al. (2017) remain valid: U^{K}_{37} indexes are not related to temperature only and should not be used as temperature proxies in Baltic Sea sediments, but the $\%C_{37:4}$ can be used as quantitative proxy for Baltic Sea surface salinity.

5. Conclusions

The combination of changes in LCA distribution and DNA sequencing in surface sediments collected along the Baltic Sea surface salinity gradient revealed, for the first time, the identity and dispersal of LCA-producing organisms in this brackish environment. Organisms from the three known groups of Isochrysidales are present in the Baltic Sea. Using $C_{39:3a}$Et, $C_{40:3a}$Et, and $C_{37:3b}$Me LCAs as end-members allowed identification of Group III, Group II, and Group I Isochrysidales, respectively.

As suggested by the LCA distributions, Group III Isochrysidales are present in the Skagerrak. The absence of Group III Isochrysidales DNA sequences was surprising. It may be related to the sub-optimized DNA amplification reactions resulting in poor recovery of LCA-producing haptophyte sequences, and thus a biased representation of the overall haptophyte community at this specific location. An unexpected Group III Isochrysidales LCA distribution in the Arkona Basin is potentially related to the advection at depth of LCAs produced by Group III Isochrysidales in the Skagerrak and the Kattegat Sea. Group II Isochrysidales, that are euryhaline, were found throughout the Baltic Sea and the Skagerrak. Group I Isochrysidales, typically found in oligohaline, lacustrine environments, were characteristic for the oligohaline, northern Baltic Sea. The presence of Group I Isochrysidales DNA sequences across the entire Baltic Sea was unexpected, but may be related to transport of environmental DNA out of the northern Baltic Sea via surface currents. However, the imprint of Group I Isochrysidales was absent in the LCA distributions of central and southern Baltic Sea surface sediments. The amount of corresponding transported Group I Isochrysidales LCAs was probably too low to be detected. This observation illustrates the importance of combining lipid analysis with DNA sequencing of surface sediments.

Recently published results on the environmental (temperature and salinity) effects on LCA distributions and indexes (U^{K}_{37} and $\%C_{37:4}$) in Baltic Sea surface sediments are not affected by the application of a new, more adapted method for LCA analysis. Two recently developed indexes (RIK_{37} and RIK_{38E}) were additionally tested in the Baltic Sea settings. The RIK_{38E} is related neither to surface temperature nor surface salinity. While the RIK_{37} is not related to surface temperature as well, a significant positive relationship with surface salinity was found in the oligohaline, northern Baltic Sea. By supplementing the dataset with available data from oligohaline lakes, a calibration curve could be produced, which reflects changes in the amount of Group I Isochrysidales relative to Group II Isochrysidales as a function of surface salinity. While this calibration still has to be tested on sediment cores, it may represent a useful salinity proxy in oligohaline environments.

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