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SURVEY OF PRESENCE OF NON-INDIGENOUS *EURYTEMORA CAROLLEEAE* IN THE GULF OF RIGA (BALTIC SEA) FIVE YEARS AFTER ITS FIRST DISCOVERY

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In the brackish ecosystem of the Gulf of Riga, the calanoid copepod Eurytemora affinis *is a key species, but recently a new non-indigenous species* Eurytemora carolleeae *was discovered in the region. In the present study, we aimed to validate the presence of* E. carolleeae *in the southern part of the Gulf of Riga five years after its first discovery. The study area is the closest region to the Rīga harbour — the main source of non-indigenous species arrival in the Gulf. Recent studies have predicted the possible potential of* E. carolleeae *invasion due to its physiological plasticity. Male and female specimens of* Eurytemora *were collected in spring, summer, and autumn of 2013, and analysed based on three morphological indicators. Despite the higher reproduction rate of* E. carolleeae*, this intrusive species does not seem to have succeeded in establishing during the five-year period after its first discovery in the Gulf of Riga, and hence does not present a threat to the native* E. affinis *population in the study area.*

Key words: *zooplankton, calanoid copepod, brackish, invasion.*

INTRODUCTION

Eurytemora affinis (Poppe, 1880) is a true estuarine calanoid copepod that inhabits brackish and fresh-waterbodies all over the northern hemisphere (e.g., Croskery, 1978; Lee and Frost, 2002). It has been described as a complex of cryptic species, because *E. affinis* populations are genetically highly divergent, though with similar morphological characteristics (Lee, 2000; Lee and Frost, 2002; Winkler *et al*., 2011; Sukhikh *et al*., 2016). Recent subsequent studies (Alekseev and Souissi, 2011; Sukhikh and Alekseev, 2013) distinguished two separate species within the complex of *E. affinis,* which launched a debate about if *E. affinis* is really a complex of cryptic or pseudocryptic species (Lajus *et al*., 2015). However, the present study refers to the native population of the Baltic Sea as *E. affinis,* instead of as a species complex, for the sake of convenience.

Eurytemora affinis is a dominating estuarine copepod in the Baltic Sea (Telesh and Heerkloss, 2004) and a key species in our study area — the Gulf of Riga (Ojaveer *et al*., 1998; Kornilovs *et al*., 2004*).* It is the main food source for the local herring (Anonymous, 2013; Livdāne *et al*., 2016) and one of the most abundant calanoid copepods controlling phytoplankton biomass during summer (Jurgensone *et al*., 2011). A recent study by Sukhikh *et al.* (2013) revealed the presence of *Eurytemora carolleeae* Alekseev and Souissi, 2011 in several sites of the Baltic Sea, including in the Gulf of Riga. *Eurytemora carolleeae* is a newly described species within the *Eurytemora affinis* complex (Alekseev and Souissi, 2011). Its invasion source was presumably from the eastern coast of North America where it is one of dominant estuarine copepods (Alekseev and Souissi, 2011). *Eurytemora carolleeae* was observed in the Gulf of Riga at low frequency (only 2–7% of morphologically analysed individuals), but analysis of DNA nucleotide barcoding of the same population did not confirm its presence. However, its presence in the eastern part of the Gulf of Finland has been confirmed by both morphological and molecular methods, where its abundance varied from 4% to 47% for females and from 2% to 50% for males of analysed *Eurytemora* individuals (Sukhikh *et al*., 2013). Therefore, the possible *E. carolleeae* invasion in the Gulf of Riga was suspected. Sukhikh *et al.* (2013) also expected the establishment of *E. carolleeae* in the Gulf of Riga in the nearest future based on "the Gause's principle (Gause, 1932) of inevitable displacement of one of the species competing for the same resource

or niche" as suggested by Alekseev *et al.* (2009). Higher reproduction rates of the North-American *Eurytemora* (Beyrend-Dur *et al*., 2009; Pierson *et al*., 2016), as well as longer lifespan of adults (Beyrend-Dur *et al*., 2009) can potentially contribute to successful invasion and establishment of the non-indigenous *Eurytemora* in the Baltic Sea, which might endanger the native population of *E. affinis*.

"Trends in arrival of new non-indigenous species" is one of HELCOM Core indicators (Anonymous, 2015) with the main goal to minimise anthropogenic introduction of non-indigenous organisms to zero. The Good Environmental Status boundary for this indicator has been set to "no new introductions of non-indigenous species per assessment unit through human activities during a six-year assessment period" (Anonymous, 2015). Application of the indicator list of present non-indigenous species has to be updated regularly. Therefore, the aim of the present study was to re-evaluate the presence of *E. carolleeae* in the Gulf of Riga five years after its first record in 2008 (Sukhikh *et al*., 2013).

MATERIALS AND METHODS

Study site. The Gulf of Riga is located in the eastern part of the brackish Baltic Sea. It is a relatively isolated basin with a northward salinity gradient. Salinity ranges between 0.5–2.0 psu in its southern areas where the largest freshwater discharge occurs, to 7.0 psu in northeast regions, but generally, salinity varies from 5.0 to 6.5 psu (Berzinsh, 1995). These low salinities are favourable for the development of *Eurytemora affinis* (Devreker *et al*., 2009). Furthermore, the Gulf of Riga is a shallow waterbody with a mean depth of 26 meters; thus, the water temperature closely follows the seasonal cycle of air temperature, reaching a maximum of 17–20 °C in upper layers during summer and freezing temperatures in winter. Consequently, a seasonal thermal stratification occurs from May to August (Kotta *et al*., 2008).

Sampling and identification. Sampling was conducted in station 119 (Fig. 1) in May, August, and November 2013. The sampling station is located close to the Rīga harbour, a hotspot of non-indigenous species arrival, as shipping is one of the main vectors responsible for species introduction in the Baltic Sea (Ojaveer *et al*., 2017).

One mesozooplankton sample was collected in each sampling event by means of vertical haul from bottom to top using a WP-2 plankton net with mesh size 100 µm and opening 0.25 m^2. The collected samples were preserved with 4% formaldehyde solution in seawater. In total, 75 females and 75 males of *Eurytemora* were randomly selected and studied, 25 individuals of each sex per sample. Morphological comparison between *E. affinis* and *E. carolleeae* was done using three indices following Sukhikh *et al.* (2013):

- for both sexes: Index 1 = length/width of the caudal rami (Fig. 2 A, B);

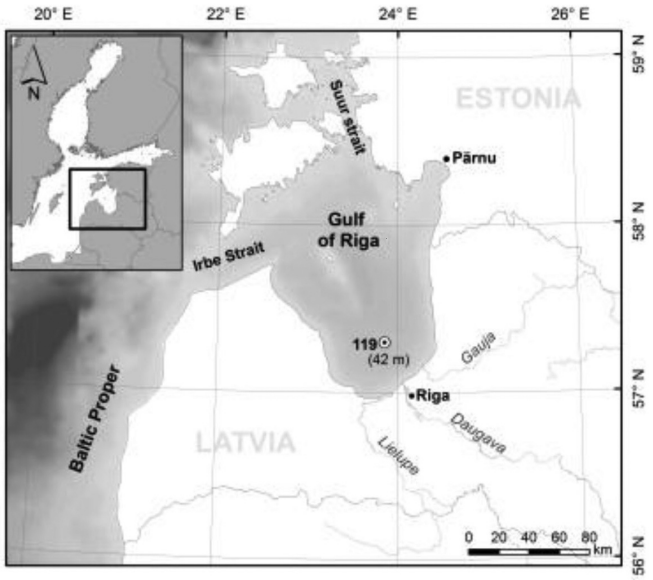

Fig. 1. Map of the study site. Sampling station 119 (N 57°18' E 23°51') is marked with a white circle with small black dot in the middle. It is approximately 42 meters deep.

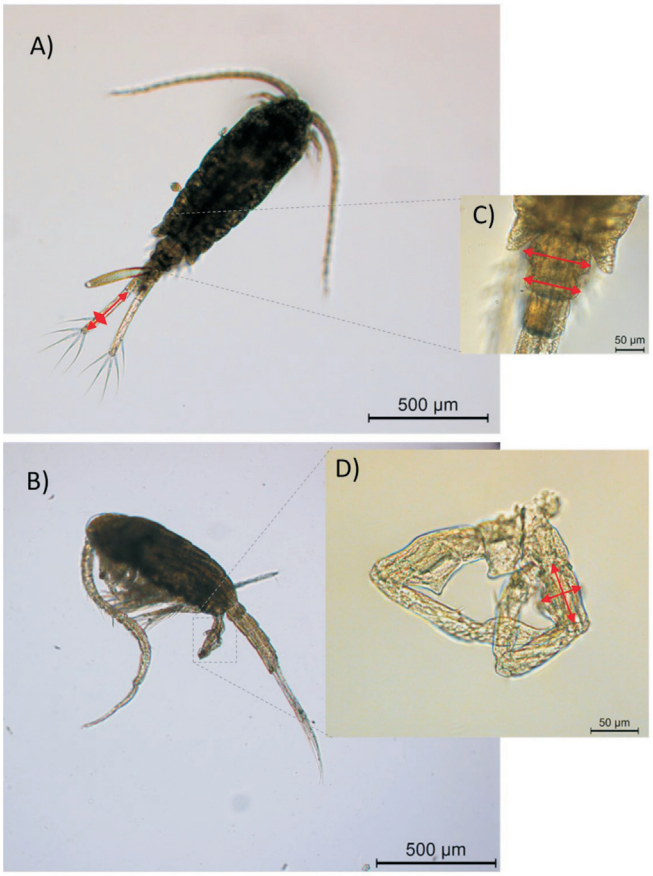

Fig. 2. Chosen morphometric indices for *Eurytemora* identification: A) adult *Eurytemora* female; red arrows indicate length/width of caudal rami (complementary Index 1, used for both sexes); B) adult *Eurytemora* male; C) female genital segment (Index 2); D) fifth pair of male swimming legs (Index 3). Red arrows indicate measured parameters.

- for females: Index 2 = anterior/posterior sides of the genital segment (Fig. 2 C);

- for males: Index 3 = length/width of the exopodite first segment in leg 5 (Fig. 2 B, D).

Firstly, photos of the morphological features needed for measurement of each parameter were taken using a compound microscope Leica DM4000 equipped with a built-in digital camera Leica DFC295 and Leica software LAS v4.1. Measurements were made with ImageJ© software (Rasband, 1997–2015). The data were analysed based on the first description of *E. carolleeae* (Alekseev and Souissi, 2011) and the study of the new species pattern in the Baltic Sea region (Sukhikh *et al*., 2013).

According to previous studies (Alekseev and Souissi 2011; Sukhikh *et al*., 2013), the main difference between *E. affinis* and *E. carolleeae* females is Index 2, which is much higher (1.35) in *E. carolleeae* compared to the native *E. affinis* (Alekseev and Souissi, 2011). In contrast, Index 1 does not seem to be a good discriminator between both species in the Gulf of Riga; *E. affinis* females have average value of 7.59 for Index 1, compared to a value of 6.06 for *E. carolleeae* in Chesapeake Bay (Alekseev and Souissi, 2011). Based on the available literature, we set an indicative value of Index 1 for *E. carolleeae* less than 6.50, but we treated it as a complementary parameter. Similarly for males, Index 1 was treated as a complementary parameter for *E. carolleeae* identification, because the caudal rami length/width ratio in *E. affinis* males from the Gulf of Riga and *E. carolleeae* males from the Chesapeake Bay overlaps even more than in females. In this case the indicative value of Index 1 was set to 9.50. Index 3 was treated as a determinant index for *E. carolleeae* detection and values higher than 1.40 strongly indicating affiliation to *E. carolleeae*. However, the most unambiguous proof of *E. carolleeae* presence is when both 2 and 3 indices deviate from the set indicative values (Table 1).

Statistical analysis. Index 1 corresponded to a normal distribution. Index 2 and Index 3 were Box-Cox transformed (Wessa, 2015). The "Outlier Labelling Rule" (Hoaglin *et al*., 1986) was applied to detect statistically significant outliers. The independent samples T-test was used to compare indicator values between seasons The one-sample T-test, evaluated with 1000 bootstrap replicates, was used to compare measured values to indicative values to approve or disapprove affiliation to *E. carolleeae* (Table 1). Shapiro-Wilk test for Normality, T-test and bootstrapping analysis was carried out in IBM© SPSS© Statistics v20.

Table 1

USED INDICATIVE VALUES FOR SPECIES DIFFERENTIATION IN THE GULF OF RIGA*

Species	Females		Males	
	Index 1	Index 2	Index 1	Index 3
E. affinis	> 6.50	< 1.35	> 9.50	< 1.40
E. carolleeae	< 6.50	> 1.35	< 9.50	> 1.40

*Values based on Alekseev and Souissi, 2011; Sukhikh *et al*., 2013.

Index 1 (for both sexes) = length/width of the caudal rami; Index 2 (for females) = width of anterior side/width of posterior side of the genital segment; Index 3 (for males) = length/width of the exopodite first segments in leg 5 (for more information about indices see Sukhikh *et al*., 2013). Index 1 was treated as a complementary index. Index 2 and Index 3 were treated as main indices. If both indices (for each sex) deviate from the set indicative values accordingly, then strong indication of affiliation to one or another species is suggested.

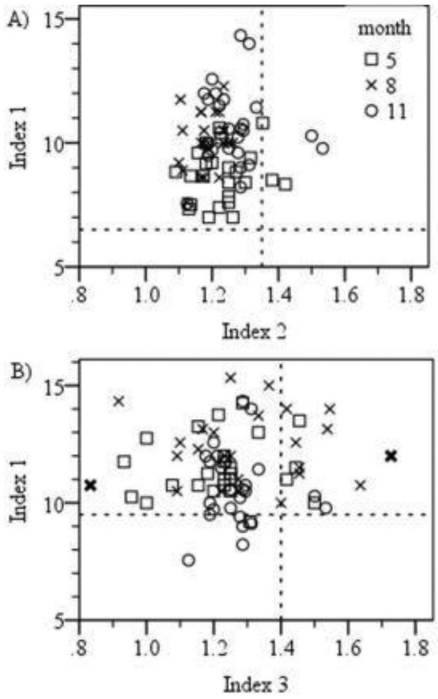

Fig. 3. Values of recommended indices (Sukhikh *et al*., 2013) for identification of *Eurytemora* sibling species (*E. affinis* and *E. carrolleae*): A) females and B) males. Index 1 (for both sexes) = length/width of the caudal rami; Index 2 (for females) = width of anterior side/width of posterior side of the genital segment; Index 3 (for males) = length/width of the exopodite first segments in leg 5 (for more information about indices see Sukhikh *et al*., 2013). Dotted lines show approximate border of indices between both species — upper left square corresponds to *E. affinis* indication; all other (but mostly two lower squares) — might correspond to *E. carrolleeae* indication (see Table 1). Bolded markers indicate statistically significant outliers ("Outlier Labeling Rule"; Hoaglin *et al*., 1986).

RESULTS

The results of morphological analysis indicated a low possibility of spread of the population of *E. carolleeae* in the Gulf of Riga (Fig. 3). In fact, only five females had a value of Index 2 higher than the set indicative value of 1.35, and for these Index 1 ranged between 8.3 and 10.8, indicating affiliation to *E. affinis*. Fifteen males reached the indicative value of 1.4 for Index 3, and one of them was determined as a statistically significant outlier (Fig. 3). Another outlier with a value just slightly above 0.8 was marked, but it was ignored in further analysis as it was well below the indicative value. However, considering the complementary Index 1, none of those fifteen males reached the indicative value needed to confirm the presence of an *E. carolleeae* male in the Gulf of Riga.

The independent samples T-test (Table 2) showed statistically significant differences between seasons for Index 1

Table 2

THE RESULTS OF INDEPENDENT SAMPLES T-TEST

			Females			Males		
			I1	I2		I1		I3
			May	May	Aug	May	Aug	
F	I1	Aug	$p < 0.001$					
		Nov	$p < 0.001$					
	I2	Aug		$p = 0.039$				
		Nov			$p < 0.001$			
M	I1	Nov				$p = 0.039$	$p < 0.001$	
Created groups			1 - May; 2 - Aug+Nov	3 - May+Nov; 4 - Aug		5 - May+Aug; 6 - Nov		7 - All data

Only significantly different groups are shown. F – Females; M – Males; I1 – Index 1; I2 – Index 2; I3 – Index 3. Last row indicates which data was pooled for each indicator (+), according to their similarity in a one-sample T-test (1-7 group No)

(for both males and females) and Index 2. *Eurytemora* females tended to have length/width ratio of the caudal rami (Index 1) significantly lower in May than in August and November, while the lowest values of Index 1 for *Eurytemora* males occurred in November. Significantly higher values of Index 2 occurred in August (Table 2). The data from seasons with non-significant differences were pooled (Table 2 last row) for comparison to target indicator values. All values of Index 3 across seasons were pooled due to non-significant variability among seasons. Again, the Index values differed significantly from the target indicator threshold values of all three indices ($p < 0.001$), reinforcing our conclusion about the low possibility of spread of *E. carolleeae* population in the Gulf of Riga.

DISCUSSION

Considering the high productivity of North American *Eurytemora carolleeae* in Chesapeake Bay (Pierson *et al*., 2016) and in the St. Lawrence estuary (Beyrend-Dur *et al*., 2009; Lloyd *et al*., 2013), its establishment in the Gulf of Riga is likely inevitable. Nevertheless, the *E. carolleeae* population has not settled and spread in the study site between 2008 and 2013, according to the results of present study. However, previous study sites were conducted in tidal zones (Beyrend-Dur *et al*., 2009; Lloyd *et al*., 2013; Pierson *et al*., 2016), which are not fully comparable to our sampling site located in the southern part of the non-tidal ecosystem of the Gulf of Riga. Indeed, the study site is an important parameter to consider, since copepod populations can differ in development between inshore and offshore waters, as demonstrated by Mouny and Dauvin (2002) for *E. affinis* in the Seine estuary. In addition, the sites where *E. carolleeae* presence was confirmed by both morphological and molecular analysis were located in coastal bays in the eastern part of the Gulf of Finland (Alekseev *et al*., 2009; Sukhikh *et al*., 2013) and this underlines the importance of the study site. The region studied in the Gulf of Finland was characterised by intensive maritime traffic and the highest ballast water discharges in the Baltic Sea (Anonymous, 2014), which might play a significant role in providing a constant and stable population of *E. carolleeae*.

The inter-annual variability in hydro-meteorological conditions can also affect *Eurytemora* abundance (Viitasalo *et al*., 1995). The year 2013 started with a harsh winter, in contrast with two consecutive mild winters of years 2007 and 2008 just before the first observation of *E. carrolleeae* in the Gulf of Riga. Furthermore, water temperature in spring 2008 was one of the highest since onset of observations (Latvian Institute of Aquatic Ecology, monitoring data, unpublished). These unique environmental conditions might have been favourable to the spread of *E. carolleeae* in that period. In addition, oceanic circulation can affect the spatial distribution of copepod species as demonstrated (St-Onge-Drouin *et al.*, 2014) for two different *E. affinis* clades present in the St. Lawrence Estuary.

Finally, individual variability of local *E. affinis* should be considered as well. Size parameters of *E. affinis* showed high seasonal, inter-annual and spatial variability in prosome and urosome length and width in the Gulf of Riga, with standard deviation ranging from 5 to 16% of the mean for females, and 6 to 13% for males (Labuce *et al*., in prep.). Considerable seasonal fluctuation in dimensions of *E. affinis* individuals of the Gulf of Riga was also described for individual body mass of copepods (Line, 1983). A similar tendency was observed in measured dimensions of caudal rami for both male and female, which disputed the applicability of Index 1 in the Gulf of Riga. In addition, seasonal variation was observed in measurements of the genital segment (Index 2), which might be related to higher reproduction rates in higher temperatures (Gasparini *et al*., 1999). Moreover, improvement in precision of measurements can be obtained by analysing symmetrical traits on both sides of the body (Lajus *et al*., 2015). In our study only one side of the body was measured for symmetrical traits (only right caudal rami; Index 1), which might have increased measurement error for Index 1 values.

As a conclusion, we deduce that there is no evidence of persistent occurrence of a *E. carolleeae* population in the Gulf

of Riga. However, inspection of coastal areas and ports should be conducted for verification, as well as more morphological indicators tested for applicability in the Gulf of Riga. Molecular analysis should also be carried out in further studies. Moreover, a regular monitoring program surveilling non-indigenous species is necessary to evaluate the presence of *E. carolleeae* and other non-native species in the study area.

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SVEŠZEMJU *EURYTEMORA CAROLLEEAE* POPULĀCIJAS KLĀTBŪTNES IZVĒRTĒŠANA RĪGAS LĪCĪ PIECUS GADUS PĒC TĀS PIRMĀ KONSTATĒŠANAS GADĪJUMA

Kalanoīdais kopepods *Eurytemora affinis* ir atslēgas suga Rīgas līča ekosistēmā, bet 2008. gada vasarā konstatēta arī svešzemju sugas *Eurytemora carolleeae* klātbūtne Baltijas jūrā, ieskaitot Rīgas līci. *Eurytemora carolleeae* ir novērtēta kā fizioloģiski plastiska suga, un nesenos pētījumos tiek norādīts uz tās iespējamo invāziju Baltijas jūrā. Šajā pētījumā tika izvērtēta svešzemju *E. carolleeae* populācijas klātbūtne Rīgas līča dienvidu daļā, proti, reģionā vistuvāk Rīgas ostai, kas darbojas kā viens no galvenajiem avotiem jaunu sugu ienākšanā. *Eurytemora* pieaugušo indivīdu taksonomiskā piederība tika izvērtēta paraugos, kas ievākti 2013. gada maijā, augustā un novembrī, izmantojot trīs morfoloģiskus indikatorus. Tomēr, neraugoties uz efektīvākiem reprodukcijas rādītajiem un garāku pieaugušo indivīdu dzīvildzi, šī pētījuma rezultāti liecināja, ka *E. carolleeae* nav spējusi veiksmīgi iedzīvoties Rīgas līcī, un tāpēc pagaidām nav apdraudējums vietējai *E. affinis* populācijai.