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Baseline paper

Cellular energy allocation in mussels (*Mytilus galloprovincialis*) from the stratified estuary as a physiological biomarker

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Abstract

This study demonstrates the usefulness of cellular energy allocation (CEA) evaluations as a physiological biomarker to infer the occurrence of natural stress in native populations of mussels inhabiting the stratified estuary (Krka River estuary, Croatia). Sampling sites were selected based on their differences in the salinity and temperature. The CEA value was calculated as a ratio between available energy (E_a) and energy consumption (E_c). Generally, higher values of E_a were recorded in June than in November, which were especially evident in the storage components (carbohydrates and lipids), while the constitutive component (proteins) remained relatively constant. The highest E_c was recorded in mussels at estuarine site compared to coastal site, which may be caused by the energetically costly maintenance of osmotic balance. Decrease in CEA recorded in estuarine mussels may ultimately result in a lower amount of energy available for growth, reproduction, or defence against other stresses (e.g. pollution).

Keywords: cellular energy allocation; natural stress; salt-wedge estuary; mussels; *Mytilus galloprovincialis*

Despite the well-established usefulness of the biomarker approach, the considerable influence of biotic and abiotic environmental factors on biomarkers could complicate the quantification and interpretation of field results (Fattorini et al., 2008; Ivanković et al., 2005; Petrović et al., 2004). Beside the stress posed by toxic substances, the natural stress caused by abiotic environmental factors affects the organisms, especially those inhabiting a highly variable environment such as an estuary (Hamer et al., 2008). The littoral zone represents a highly variable environment that significantly affects the metabolism of marine molluscs because of the incidence of cyclic exposure to air and consequently, intermittent hypoxia/anoxia that causes important changes in the energy metabolism of animals. Organisms that live in such extreme environmental conditions might fail to reproduce or even die due to natural environmental stress (thermal stress, salinity stress, etc.). Nevertheless, mussels (*Mytilus* sp.) are able to cope with the changes in abiotic factors (salinity, temperature, dissolved oxygen) owing to their ability to adapt to a wide range of salinities and their efficient respiratory physiology (Hawkins and Bayne, 1992; Zandee et al., 1986).

When organisms live in suboptimal environments, there will be a cost of dealing with stress in terms of metabolic resources. The energy available for growth, based on energy budget analysis rather than on direct measurement of growth itself, therefore provides a sensitive measure of stress in an organism. Growth as a process represents an integration of major physiological responses and specifically the balance between processes of energy acquisition (feeding and assimilation) and energy expenditure (metabolism and excretion). The physiological energetics-based approach is usually referred to as Scope for Growth (SfG) and has been used extensively with marine mussels (Widdows and Johnson, 1988). De Coen and Janssen (1997) have proposed a biochemical alternative to the physiological SfG, referred to as cellular energy allocation (CEA). The concept of this approach is to quantify the available

energy reserves and energy consumption at a cellular level of biological organisation and to incorporate all components into a net cellular energy budget of the organism. The CEA approach has proven to be ecologically relevant as cellular effects have been linked to higher levels of biological organization (De Coen and Janssen, 2003; Smolders et al., 2004). So far, CEA has been applied in laboratory and field studies in freshwater systems using daphnia and zebra mussel (Smolders et al., 2004), in estuarine systems using *Neomysis integer* (Verslycke et al., 2004; Erk et al., 2008), in polar marine system using Arctic benthic amphipods and bivalves (Olsen et al., 2007) and in pelagic ecosystems using transplanted *M. edulis* (Smolders et al., 2006). To our knowledge, no studies on CEA as an indicator of natural stress factors have been reported so far using indigeneous mussels *M. galloprovincialis* in estuarine ecosystem.

Therefore, the main goal of the present study was to investigate the potential use of the CEA methodology in indigenous mussels *Mytilus galloprovincialis* as a research tool to study the natural stress posed by strong fluctuations in salinity occurring in the Krka River estuary.

The estuary of the karstic river Krka is a salt-wedge, highly stratified estuary, located in the central part of the eastern Adriatic coast in Croatia (Fig. 1). It is a typical example of a stratified estuary with a fresh/brackish surface layer moving seawards and a bottom seawater layer, as counter-current, moving upward. This estuary is regarded as fairly unpolluted (Cukrov et al., 2008; Omanović et al., 2006). The upper part of Krka River is located in the national park without any significant contamination sources. The input of terrigeneous material in Krka River is extremely low. In the upper part of the estuary, decomposing freshwater phytoplankton, which develops in Visovac Lake, situated above the waterfalls, is the main nutrient source (Cetinić et al., 2006). In the estuary's middle reach, the town of Šibenik was the only source of direct anthropogenic eutrophication until the year 2006. Since 2006 wastewater treatment plant is operating in Šibenik and the treated municipal wastewater

is disposed by means of the submarine outfall (the discharge is located about 3 km off the southern cape of the island Zlarin, Fig. 1). Consequently, from 2006 till today the water quality was greatly improved in the Šibenik harbour.

Two sampling sites were selected in this area based on their differences in abiotic factors, primarily in the salinity. Salinity/temperature (S/T) data for period from 1999 – 2008 show different degree of variations at selected sampling sites (Fig. 2). Accordingly, these sites were characterised as the coastal site with lower S/T variations (Zablaće) and the estuarine site with larger S/T variations (Martinska) (Fig. 1).

The indigenous mussels (*Mytilus galloprovincialis*) were collected from coastal rocks or concrete embankment structures between 0.5 and 1 m below the sea surface. The samplings were performed in June and November 2007 and 2008. The digestive gland was selected as a target organ since it serves as storage of energy reserves and represents a central detoxification site of the organism. The biometric measurements, sex determination and dissection of digestive gland were performed immediately after collection at the marine station located at Martinska. The tissues were stored in liquid nitrogen and transported to the laboratory in Zagreb for further analysis.

CEA was measured according to Verslycke and Janssen (2002) with minor modifications. Protein content was measured by the method described by Bradford (1976) using bovine serum albumine as a standard. Carbohydrate content was analyzed by phenol-sulfuric acid method (Dubois et al., 1956) using glucose as a standard. Lipids were extracted following the method of Bligh and Dyer (1959) and lipid concentrations were calculated by reference to standards of tripalmitine in chloroform. The different energy reserve fractions (lipid, protein, carbohydrate = available energy, E_a) were transformed into energetic equivalents using their respective energy of combustion (39500 mJ/mg lipid, 24000 mJ/mg protein, 17500 mJ/mg glycogen) (Gnaiger, 1983). The energy consumption (E_c) was estimated by measuring the

activity of the mitochondrial electron transport system (ETS) according to Owens and King (1975). The quantity of oxygen consumed, as derived from the ETS data, was transformed into energetic equivalents using the oxyenthalpic equivalents for an average lipid, protein and carbohydrate mixture (484 kJ/mol O_2) (Gnaiger, 1983).

The E_a, E_c and CEA value were calculated as described by Verslycke and Janssen (2002):

E_a (available energy) = $E_{carbohydrate} + E_{lipid} + E_{protein}$ (mJ/mg ww)

E_c (energy consumption) = ETS activity (mJ/mg ww/h)

CEA (cellular energy allocation) = E_a/E_c

From this, it can be evident that a decrease of CEA indicates either a reduction in available energy or higher energy expenditure, both resulting in a lower amount of energy available for growth or reproduction.

Measurements of lipid, carbohydrate and protein energy content and electron transport system (ETS) activity were performed in individual organisms. Each parameter in the individual sample was measured in replicate. The gender of mussels was determined (Jabbar and Davies, 1987) and only female mussels were analyzed.

All statistical analyses were performed with the software package SigmaStat for Windows Version 3.5. Differences in lipid, protein, carbohydrate energy contents and differences in ETS activities between sampling sites were tested using t-test. Also t-test was used to detect the differences between June and November samplings. The tests were performed at the probability level of 0.05 or the probability levels are indicated in the figures.

In this study the biochemical measurements were performed only on female mussels in order to avoid the differences between sexes. Evidences for such differences have been reported in the literature. Livingstone (1981) demonstrated that the increase in glucose-6-phosphate dehydrogenase activity that occurred in the mantle tissue during the winter months was

confined to female mussels. Zaba and Davies (1980) found that during the spawning period mantle tissue slices from female mussels metabolised glucose twice as rapidly as those from males per unit tissue weight. Therefore, it was important to take this fact into account in order to reliably assess the changes in CEA caused by natural stress. By transforming the measured concentrations of total carbohydrates, proteins and lipids into their energetic equivalents, it was possible to compare the relative contribution of carbohydrate, lipid and protein contents of digestive gland to its total energy budget. The energy contents of total carbohydrates, proteins and lipids measured in 2007, 2008 and 2010 are presented in Table 1.

Calculated equivalents of the lipid and protein components were quantitatively the most important energy sources (Table 1). Energy contents of total proteins had shown the most conservative behaviour compared to energy contents of carbohydrates and lipids. In all studied seasons and years the differences in energy contents of total proteins between the sampling sites as well as between the sampling seasons were not significant (Table 1).

Significantly higher energy content of total carbohydrates in June than in November was recorded at both sites Zablaće and Martinska in years 2007 and 2008 (t-test, $p<0.05$, Table 1). In general, the energy content of total lipids was higher in digestive glands of mussels sampled in June than in November in both studied years. Nevertheless, this difference was significant in mussels from both sampling sites in the year 2007, while in 2008 this difference was significant in mussels from Martinska (Table 1). Higher values in energy contents recorded in June, that were especially evident in the storage components (carbohydrates and lipids) can be the consequence of increased food availability which occurred after the spring phytoplankton bloom. Indeed, seasonal distribution of dissolved carbohydrates measured in the seawater, indicated that significant accumulation up to 1 mg C/L occurred in summer as a consequence of increased production of carbon-rich phytoplankton materials under nitrogen-depleted conditions (Tepić et al., 2007). Moreover, in the phase of the reproductive cycle

before the beginning of the gametogenesis (i.e. in the late spring until early summer), the storage components are accumulated in the digestive gland. The storage tissues of bivalves undergo seasonal variations in biochemical composition and in their cellular structure in relation to reproductive cycle. In the phase of nutrient storage the reserves are deposited in the form of triglycerides in the digestive gland (Mathieu and Lubet, 1993). This could also be the probable cause of higher concentrations of total lipids measured in June than in November.

Nevertheless, using digestive gland in CEA analysis can have the advantage over using the whole soft tissue. Due to the high contribution of mantle tissue and its prominent variability in the biochemical composition depending on the phase in reproductive cycle, the changes in CEA caused by natural stress could be masked. It has been shown that various aspects of the relationship exist between gametogenesis and the utilization of glycogen and protein reserves in the mantle tissue of *M. edulis* (Bayne et al., 1982).

Since the lipid contents give the highest contribution in energy equivalents (39500 mJ/mg), the trends of total E_a, which is a sum of energy contents of carbohydrates, lipids and proteins actually reflected the trends of lipids. For E_a determined in digestive glands of mussels in all studied years and seasons there were no differences between the sampling sites, with the exception in November 2008 (Fig. 3). Total available energy (E_a) was higher in June than in November at both sampling sites, but not significantly (Fig. 3).

Energy consumption (E_c) was significantly higher in mussels living at the estuarine site Martinska than at the coastal site Zablaće (Fig. 3). Since Martinska was the site with high salinity fluctuations, mussels living at this site were actually exposed to more demanding environment. To help reduce the rate of associated changes in cell volume, mussels respond immediately by closing the shell. Thus, following a sudden change in salinity, physiological rates of feeding and metabolism are initially depressed (Bayne et al., 1976). Besides, mussels are osmoconformers, which means that they maintain their internal salinity such that it is

always equal to the surrounding seawater. They maintain the volume of cells relatively constant by actively regulating their internal concentration of free amino acids and ions to match the osmolarity of the environment (Lange, 1972). These regulative processes are energetically costly. Thus, high energy consumption detected in mussels from Martinska may be caused by the energetically costly maintenance of osmotic balance.

In 2007 sampling the mussels from Zablaće had significantly higher CEA values in November than in June, while mussels from Martinska had significantly lower CEA in November than in June (Fig. 3). In 2008 sampling, there were no significant differences in CEA values between June and November at both sites (Fig. 3). Although we observed certain variability in components of available energy (E_a, Table 1) and in energy consumption (E_c, Fig. 3), the calculated CEA values showed consistency depending on the sampling site. Consistently higher CEA values were recorded in mussels at coastal site and consistently lower CEA values at estuarine site (Fig. 3). These results unambiguously show that the estuarine environment represents a very demanding environment in terms of energy needed for maintenance of life.

CEA was used to elicit the effects of environmental stress on an organism's energy status and general fitness, and it differs from other approaches in that it is based on the use of energetics at a cellular level of biological organisation. It has been used in pelagic ecosystems to evaluate the effects of exposure along marine pollution gradients in cage exposed *Mytilus edulis*, in which the integrative nature of energy budgets as an ecotoxicological endpoint has been shown (Smolders et al., 2006). At one caging site the decrease in CEA detected was not supported by the available contaminant data, but was possibly caused by its location away from the nutrient source.

Similarly to *M. galloprovincialis* in our study, estuarine mysid species *N. integer* from the Scheldt estuary, had a higher CEA in spring than in winter. It has been indicated that *N.*

integer allocated more energy towards their energy reserves in spring (Verslycke et al., 2004). The observed similar trends in different animal species highlight the cosmopolitan nature of CEA as a physiological biomarker and support its universal application as an ecotoxicological endpoint. On the other hand, the same authors have found that CEA decreased in the more upstream locations, but this decrease was not connected to the decrease in salinity but to the pollution especially at the sampling site Antwerp (Verslycke et al., 2004). Since the Scheldt estuary is highly polluted, the comparison with more pristine estuarine environment such as Krka estuary would not be justified in this case. In addition, in laboratory study testing effect of different salinities on CEA in *N. integer*, CEA value was significantly higher at 16 (isoosmotic point) than at 5 (hypo-osmotic medium) and 25 psu (hyper-osmotic medium) at the end of 7-day exposure (Erk et al., 2008). From these results it can be deduced that CEA values are decreased in animals living in the sub-optimal or demanding environments, which is corroborated by the results of our present study.

In summary, based on CEA values, a clear difference was observed between the coastal (CEA values in the range from 292 to 405) and estuarine (CEA values in the range from 193 to 262) mussels. Since the decrease in CEA value indicates a lower amount of energy available for growth, reproduction, or defence against pollution, the decrease in CEA found in the estuarine mussels compared to the coastal mussels may implicate their susceptibility to stress and it may be a sign of the vulnerability of the estuarine ecosystems. The present study represents a baseline for the use of this approach in natural ecosystems, and in the future a long-term field study would be crucial to the successful use of physiological biomarker such as the CEA.

Acknowledgement

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Figure captions

Figure 1.

Study area of the Krka River estuary with indicated sampling sites (coastal site Zablaće and estuarine site Martinska.

Figure 2.

Salinity and temperature (S/T) data for the period from 1999 – 2008 at the selected sampling sites: (A) coastal site Zablaće and (B) estuarine site Martinska.

Figure 3.

(A) Total energy available (E_a), (B) total energy consumption (E_c) and (C) cellular energy allocation (CEA) in digestive gland of *M. galloprovincialis* determined at two sampling sites in Krka River estuary (mean values and standard deviations are presented; significant differences between the sampling sites are indicated with probability levels: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Table_1

Table 1.

Available energy fractions in digestive gland of mussel *M. galloprovincialis* (data are shown as mean ± standard deviation; [a,b] - indicate significant differences between June and November in the same year; t-test, $p<0.05$)

Site	Sampling	Number of individuals	$E_{(carbohydrates)}$ $mJ/mg_{w.w.}$	$E_{(proteins)}$ $mJ/mg_{w.w.}$	$E_{(lipids)}$ $mJ/mg_{w.w.}$
ZABLAĆE (coastal site)	Jun/2007	7	192.25±14.50[a]	1533.61±159.50	4051.15±555.82[a]
	Nov/2007	7	129.57±48.38[b]	1591.51±171.83	2710.51±666.30[b]
	Jun/2008	8	373.56±80.80[a]	1509.81±122.43	3536.44±1217.80
	Nov/2008	6	182.54±78.02[b]	1654.56±152.49	2553.51±337.49
MARTINSKA (estuarine site)	Jun/2007	6	244.83±93.75[a]	1331.55±180.14	4380.78±1066.79[a]
	Nov/2007	6	73.66±32.88[b]	1552.15±208.89	2697.36±277.43[b]
	Jun/2008	10	218.53±99.96[a]	1404.29±129.42	3212.27±1023.75[a]
	Nov/2008	5	76.92±22.85[b]	1490.21±124.53	2007.13±495.73[b]

Figure_1
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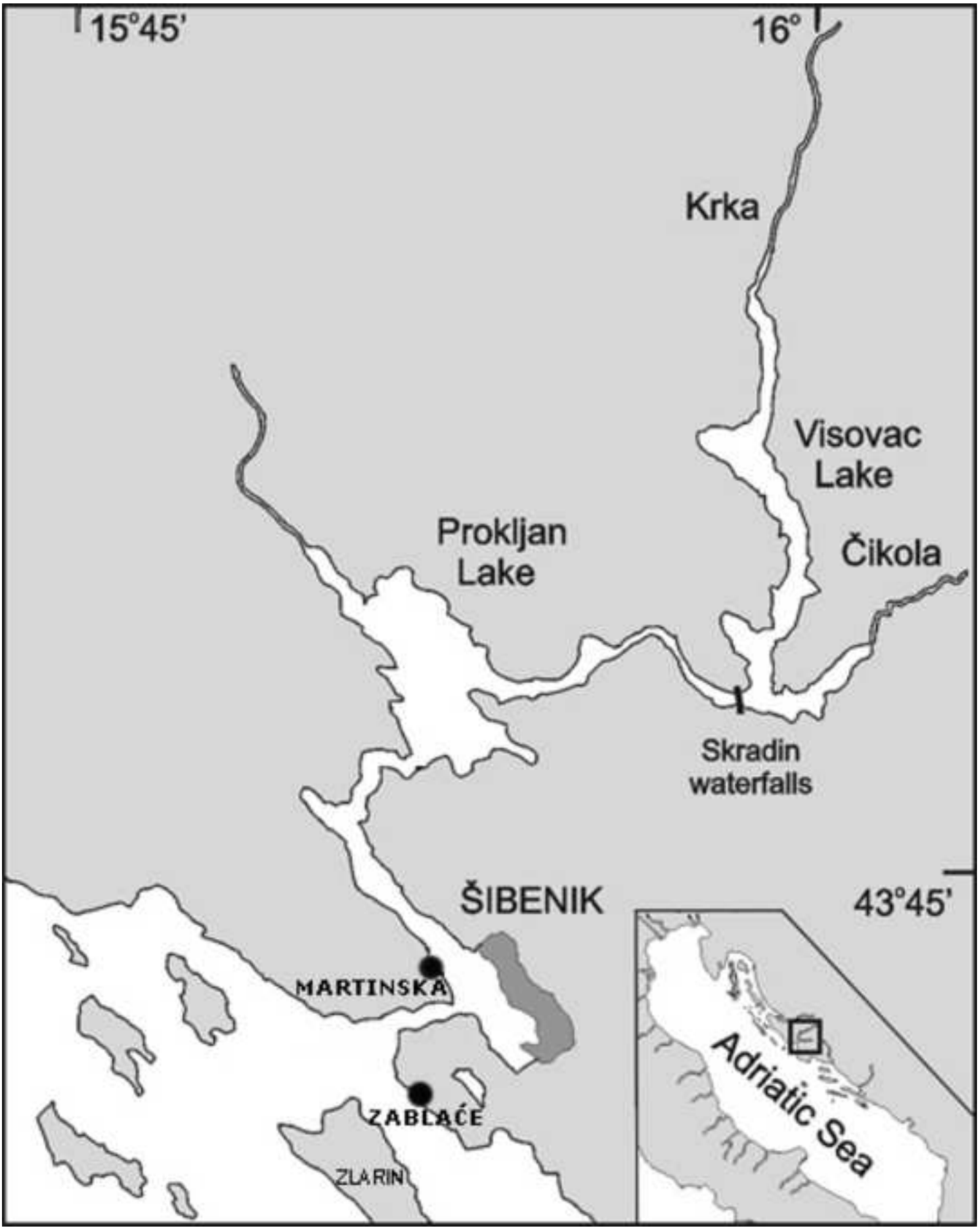

Figure_2A

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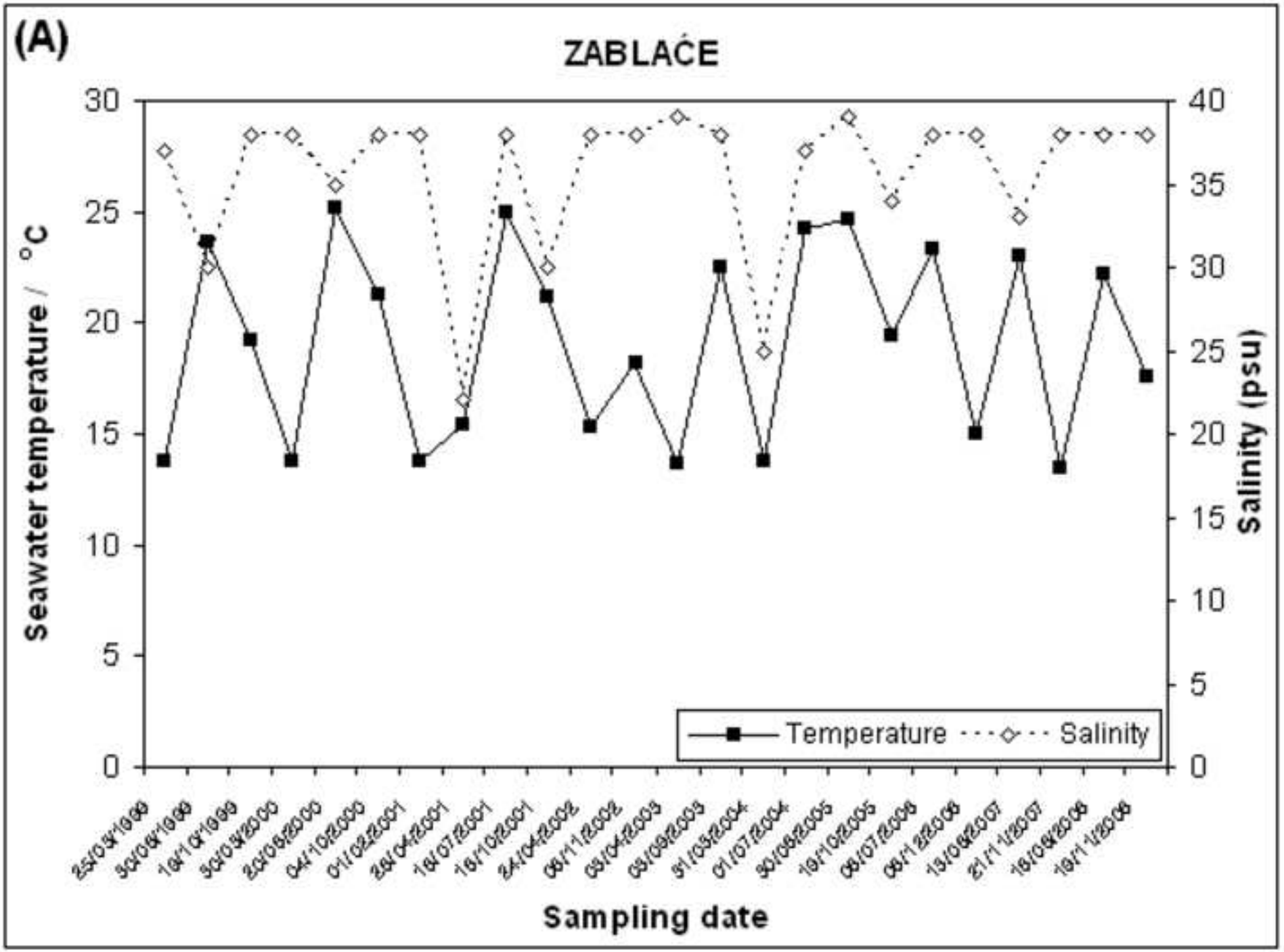

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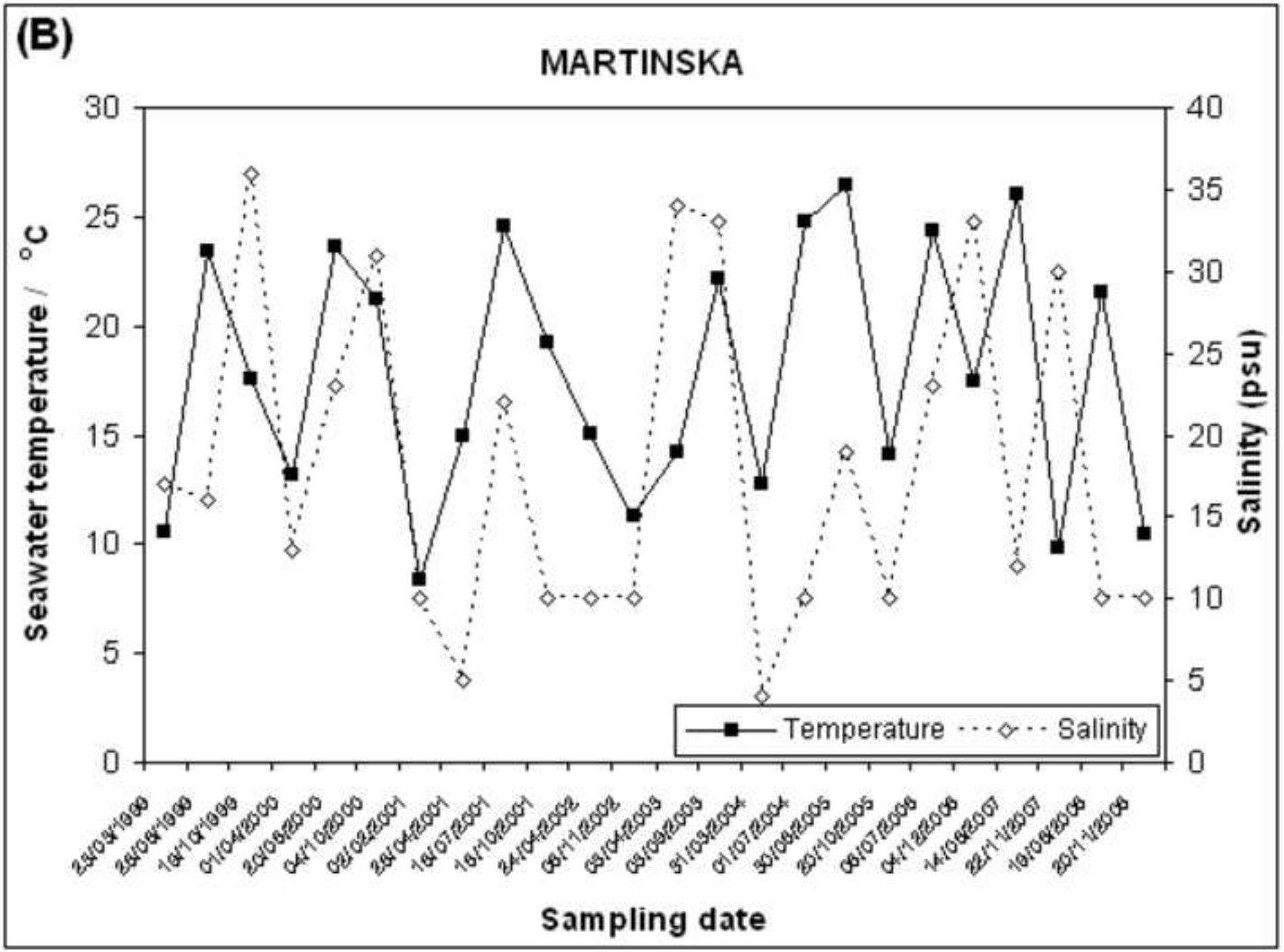

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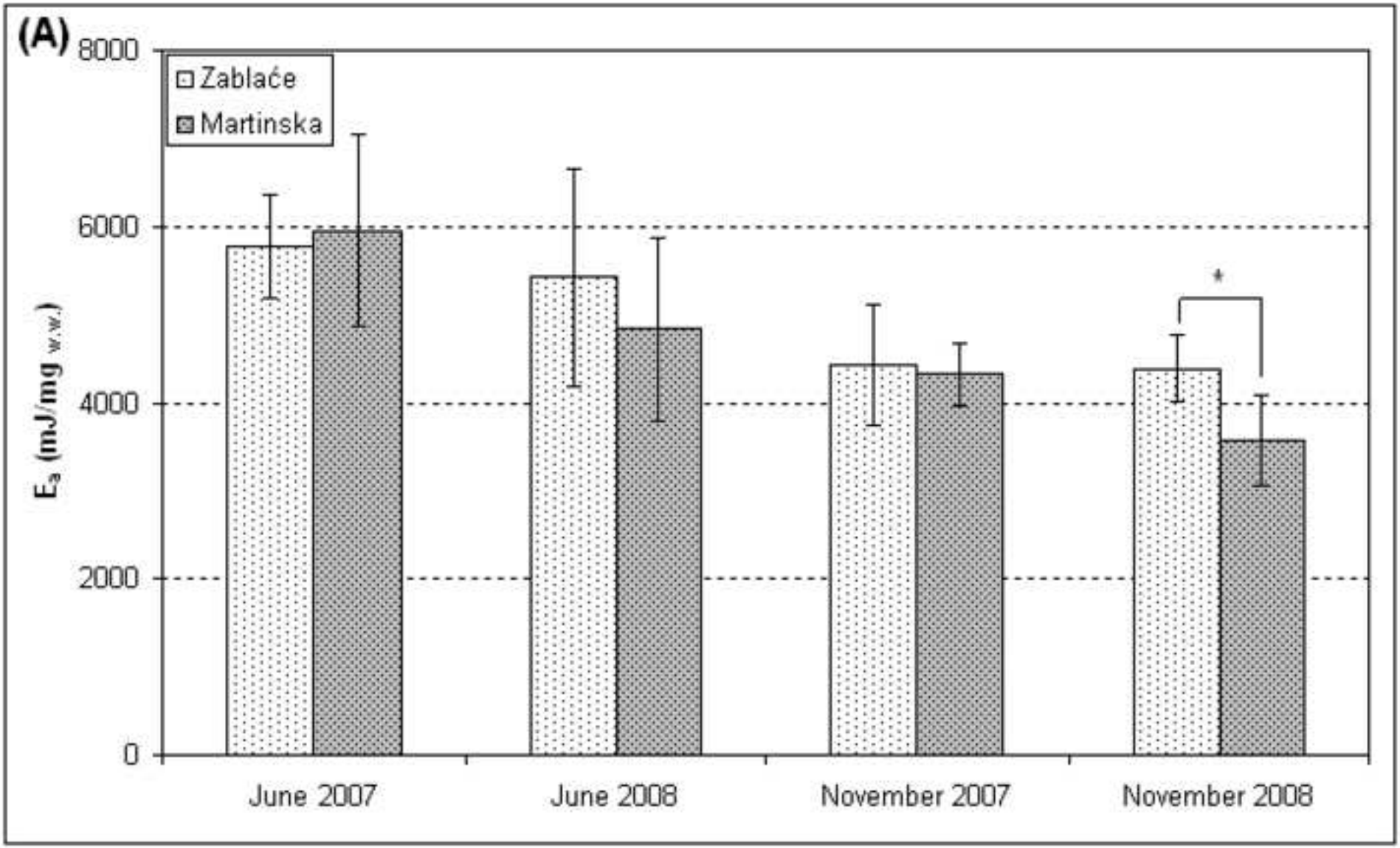

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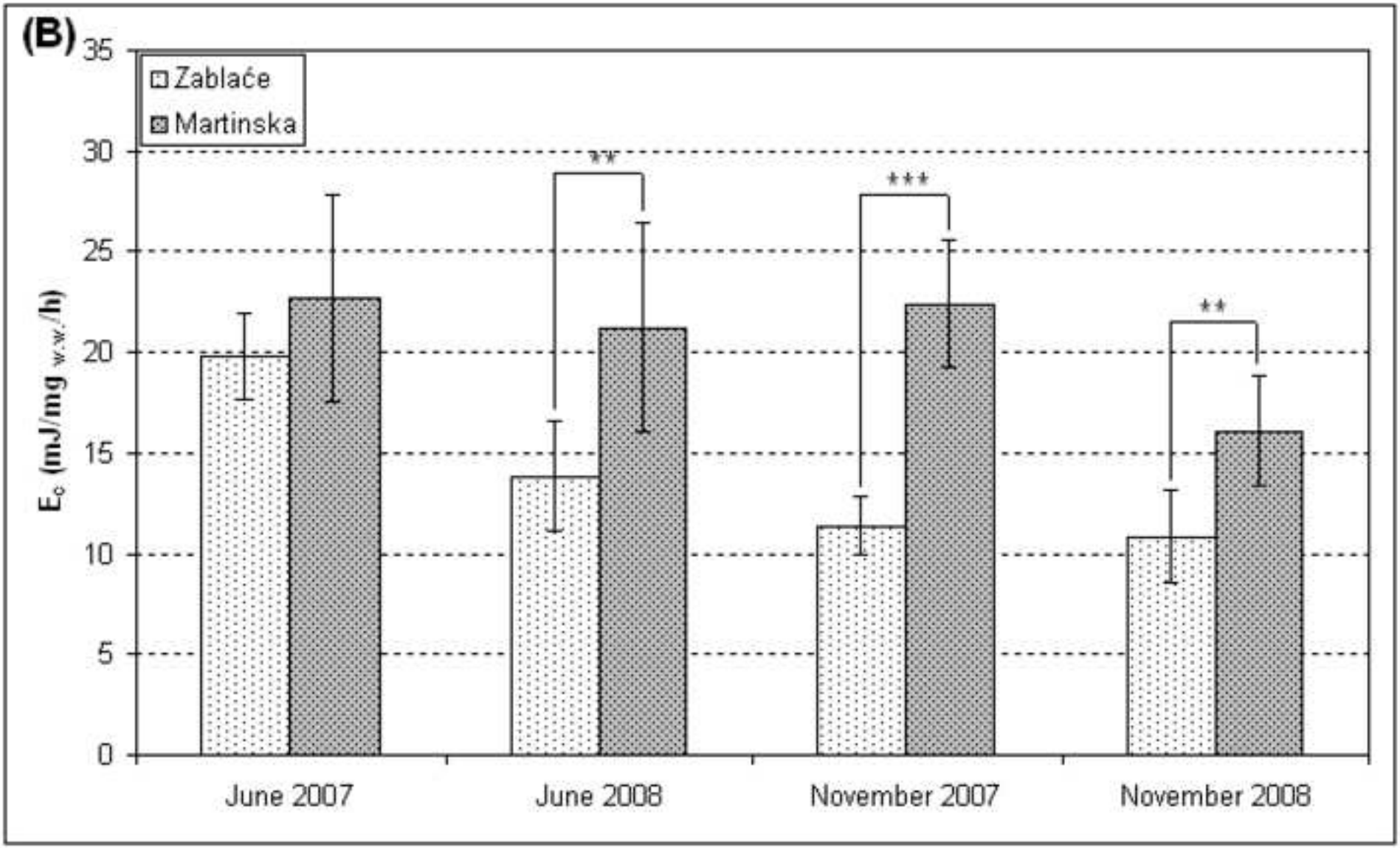

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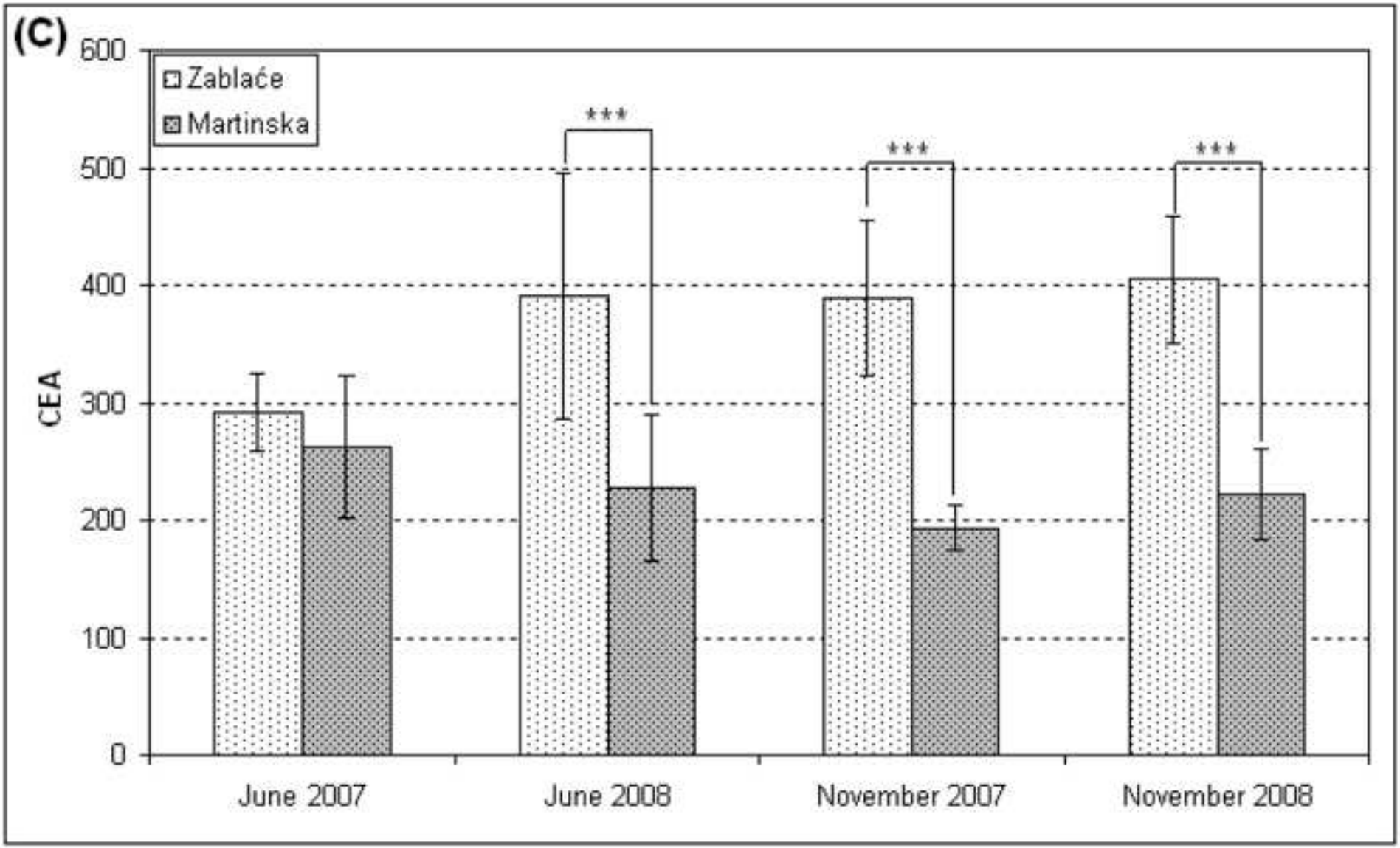
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