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The accumulation of the indicator bacteria *Escherichia coli* in mussels (*Mytilus galloprovincialis*) and oysters (*Ostrea edulis*) under experimental conditions

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The simultaneous effects of temperature and salinity on the accumulation of Escherichia coli (E. coli) *in mussels* (Mytilus galloprovincialis) *and oysters* (Ostrea edulis) *were studied under experimental conditions with different concentrations of* E. coli *in seawater. The experiments were carried out in 3000 L tanks, within the natural range of temperature* (*12°C, 18°C and 24°C*) *and salinity* (*32 psu and 37 psu*) *in the coastal areas of the Adriatic Sea. Within the achieved range of* E. coli *concentrations in seawater* (*4x10-4.6x10³ CFU/100 ml*), *bacteria accumulated rapidly in the bivalves, reaching the highest concentrations (plateau) after 1 h in mussels and 2 h in oysters. Under the same concentrations of* E. coli *in seawater, a significantly higher plateau was reached in mussels than oysters. Significant correlations between the* E. coli *concentration in seawater and bivalves* (*CR; concentration ratio*) *were found. The results clearly showed that CR was controlled by temperature and salinity of seawater. Changes in temperature had a strong effect on CR fluctuation, whereas in oysters an effect of changes in salinity on CR variation was also found only in oysters. The interaction between temperature and salinity was statistically significant and suggested that their simultaneous effects on CR were significantly greater than when each of these factors acted independently. The highest* E. coli *concentrations in mussels and oysters exceeded the concentrations in seawater by an order of magnitude at least, while the CR in mussels exceeded the CR in oysters by 7.7- to 38.5-fold.*

Key words: *Mytilus galloprovincialis*, *Ostrea edulis*, *Escherichia coli*, concentration ratio, temperature, salinity

INTRODUCTION

As filter-feeding organisms, raw and undercooked bivalves have, for many years, been recognised as an important vector of many viral and bacterial pathogens (RIPPEY, 1994). Since the isolation of pathogens is a difficult, expensive and time-consuming process, indicator microorganisms are used as an index of human-specific faecal pollution and to classify the waters from which bivalves are harvested. Due to simple methods for its isolation in bivalves (DONOVAN *et al.*, 1998) and a positive correlation with other pathogens (HOOD, 1983), *E.*

coli has been recognised as an acceptable indicator bacteria.

The accumulation of *E. coli* and other enteric bacteria in bivalves is a dynamic process. It's related to the filtration rate, to the bacterial content of the ambient water and to the filtration efficiency of the gills (KUEH, 1987). Although there is no consensus in the literature on the process of filtration in bivalves, many studies have shown that it is a physiological process regulated by many ecological factors, primarily by temperature (HAURE *et al.,* 1998; KITTNER & RIISGÅRD, 2005), but also by salinity (COLE & HEPPER, 1954; RAJESH *et al.,* 2001), particle size (TAMES & DRALL, 1955; DUPUY *et al.,* 2000) and concentration (SHULTE, 1975; ŠOLIĆ *et al.,* 2007). Since bivalves are ectothermic organisms, temperature has been recognised as a major determinant of their physiological status (BAYNE, 1976), and its separate effect on the accumulation of enteric bacteria in bivalves has been assessed in many studies. However, the interactive effects of temperature and other factors, primarily salinity, on the accumulation of enteric bacteria have been poorly investigated. The aim of the present research was to study the simultaneous effect of temperature and salinity on the accumulation of the indicator bacteria *Escherichia coli* in mussels (*Mytilus galloprovincialis*) and oysters (*Ostrea edulis*) under experimental conditions with different concentrations in seawater.

MATERIAL AND METHODS

Bivalves for experiments were taken from the shellfish garden, classified as class A (≤ 230 MPN *E. coli*/100 g) (EU REGULATION, 2004), kept in tanks with continuously flowing natural seawater until the starting of the experiments. Bacterial suspensions were prepared from a pure culture of *E. coli* ATCC 35218, 18 hours before the initiation of the experiments. *E. coli* were incubated 8 hours in mineral-modified glutamate broth (MMGB) at temperature 36.5°C and then kept in phosphate buffered solution at temperature 4°C in concentration of about $1x10^5$ CFU/ml.

The experiments were carried out in 3000 L tanks (195 cm diameter, 120 cm high), protected from direct sunlight exposure. After the suspensions of *E. coli* were added, the seawater was mechanically stirred for 10 minutes, then maintained by bubbling air into the tanks. Bivalves were placed in the tanks in metal baskets containing 10 mussels and 10 oysters each, and then sampled in 1 hour intervals by taking one basket from each tank. The *E. coli* concentration in tanks was determined by sampling the seawater at four diametrical points at the beginning and at the end of the experiments. Experiments were carried out with 12 different conditions for each species, and in three replicates. Experiments were performed seasonally, at three temperatures (12°C - mean winter temperature, 18°C - mean spring and autumn temperature and 24°C - mean summer temperature), two salinities (32 psu - common salinity in estuarine areas where shellfish farms are often located and 37 psu - typical salinity in coastal seawater) and two *E. coli* concentrations in tanks ("low" - about $1x10^2$ CFU/100 ml and "high" - about $2x10^3$ CFU/100 ml). Lower salinity (32 psu) was obtained by gradually reducing normal salinity (1 psu a day) by adding of fresh water, and kept stable 7 days before initiation of the experiments.

The concentration of *E. coli* in suspension was determined by the epifluorescent microscopy method (PORTER & FEIG, 1980). The number of culturable *E. coli* in suspension and in seawater tanks was determined by the membrane filtration (MF) method according to HRN ISO 16649-1 and in shellfish by the most probable number (MPN) method according to HRS ISO/TS 16649-3.

RESULTS AND DISCUSSION

The average size of mussels in this study was 71.8±4.2 mm; oysters were 76.3±5.3 mm in size. The variations in size of bivalves were not statistically significant ($P<0.05$), so the effect of size on filtration activity in bivalves could be excluded. A positive correlation between filtration rates and bivalve size was found in earlier studies with mussels (*Mytilus edulis*) (WINTER,

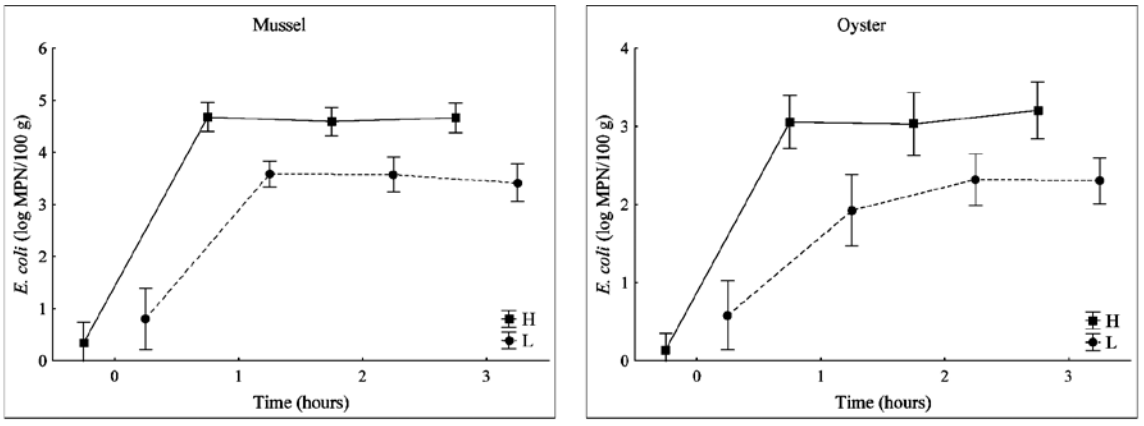

Fig. 1. Uptake of E. coli *by mussels and oysters as a function of time under conditions of "high" (H) and "low" (L)* E. coli *concentrations in tanks (mean values ± SD)*

1973; RIISGARD & MOHLENBERG, 1979) and oysters (*Crassostrea madrasensis*) (RAJESH *et al.,* 2001). In their study on mussels (*Mytilus galloprovincialis*), OKUMUŞ *et al.* (2002) found that although mussel size did not have a clear effect on cell consumption, filtration rates seemed to increase with mussel size.

The greatest increase and the highest *E. coli* concentrations were observed in the first hour of the experiment (Fig. 1). A similar rapid bacteria accumulation in mussels at the initial stage of exposure (1-2 hours) was also found by BIRBECK & MCHENERY (1982) and MARINO *et al*., (2005). In oysters, the highest increase in *E. coli* concentrations was observed in the first hour, but the highest *E. coli* concentrations were reached in the period of 2-3 hours (Fig.1). A slower achievement of the maximum concentration of coliform bacteria in oysters (*Crassostrea gigas*) was also found by BERNARD (1989) in experiments performed in seawater filtered through a 500 µm filter. After reaching the highest values (plateau), *E. coli* concentrations oscillated slightly in both bivalves. Sustained *E. coli* concentrations near the plateau were probably the result of pseudofaeces production (FOSTER-SMITH, 1975) or a reduction in the filtration rate in response to increasing food concentrations, thus maintaining ingestion rates at a constant level without producing pseudofaeces (SPRUNG & ROSE, 1988). WINTER (1973) suggested that the blue mussel (*Mytilus edulis*) might adjust feeding rates by switching the ciliary pump on and off for some period of time.

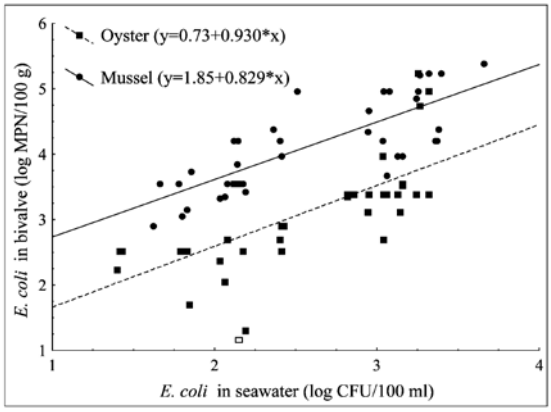

Fig. 2. Relationship between the concentration of E. coli *in bivalves and seawater (R^2=0.62 for mussels and R^2=0.48 for oysters)*

The highest *E. coli* concentrations in both bivalve species were significantly higher ($P<0.001$) at higher *E. coli* concentrations in seawater, which were in the range of $8.8x10^2$-$4.6x10^3$ CFU/100 ml, compared with lower *E. coli* concentrations in seawater, which were in the range of 4x10-$3x10^2$ CFU/100 ml. By comparing the highest concentrations of *E. coli* in both bivalve species with the *E. coli* concentrations in ambient seawater, regardless of temperature and salinity, a significant positive correlation was found (Fig. 2).

Although the increase to the highest *E. coli* concentration in bivalves as a function of *E. coli* concentration in seawater was slightly more rapid in oysters than in mussels, these reached,

on average, one order of magnitude higher concentrations than oysters. A positive correlation between *E. coli* concentrations in shellfish and ambient seawater was also found by CABELLI & HEFFERNAN (1970). Comparing the concentrations of faecal coliforms in mussels (*Mytilus galloprovincialis*) and ambient seawater under natural conditions, ŠOLIĆ *et al.* (1999) found a very similar slope (0.79).

By performing t-tests, a statistically significant effect (P>0.05) of variations in the concentrations of *E. coli* in seawater was not found on the ratio between *E. coli* concentration in bivalves and seawater (CR; concentration ratio). The results of two-way ANOVA revealed a separate and interactive effect of temperature and salinity on CR. In mussels, variations in temperature had a significant effect on changes in the CR, while variations in salinity had a significant effect only in the interaction with temperature. In oysters, variations in temperature had more of an effect than variations in salinity, while a statistically significant interaction between temperature and salinity suggested that their simultaneous effect on CR was significantly greater than their separate effects (Table 1). Variations in the *E. coli* concentrations in seawater had no significant effect on CR in either mussels or oysters (Table 1). Analysis of the results according to different temperatures and salinities showed a strong correlation (R^2=0.75-0.96) between the highest *E. coli* concentrations in bivalves and the *E. coli* concentration in seawater. It was clear that the relation between these two concentrations was not a simple function of separate effects of temperature and salinity, but also of their interactive effect (Fig. 3).

In mussels, absolute values of CR as a function of temperature and salinity were in the range of 12-84, with an average value of 47.7. At a temperature of 18°C, a positive correlation between CR and salinity was found, while at higher and lower temperatures, the correlation was negative. The highest CR values at a salinity of 37 psu were found at 18°C, while at a salinity of 32 psu, the highest CR values were found at 24°C. In oysters, there was a relatively wider range of CR values (1.56-40.8), and the average value of 9.5 indicated the prevalence of a significantly lower CR. At a temperature of 24°C, a negative correlation was found between CR and salinity, while at lower temperatures, the

Table 1. Results of two-way ANOVA comparing the effects of temperature, salinity and concentration of E. coli *in seawater on the concentration ratio (CR)*

Source of variation	SS	df	MS	F	P
Mussel:					
Temperature (T)	4443,97	2	2221,98	4,8597	0,016903*
Salinity (S)	22,81	1	22,81	0,0499	0,825139
Interaction (TxS)	20507,17	2	10253,59	22,4257	0,000003**
Error	10973,40	24	457,23	4,8597	0,016903
Oyster:					
Temperature (T)	2999,482	2	1499,741	14,92890	0,000061**
Salinity (S)	979,888	1	979,888	9,75412	0,004623**
Interaction (TxS)	3178,233	2	1589,117	15,81857	0,000042**
Error	2411,014	24	100,459		

* P<0.05; ** P<0.01

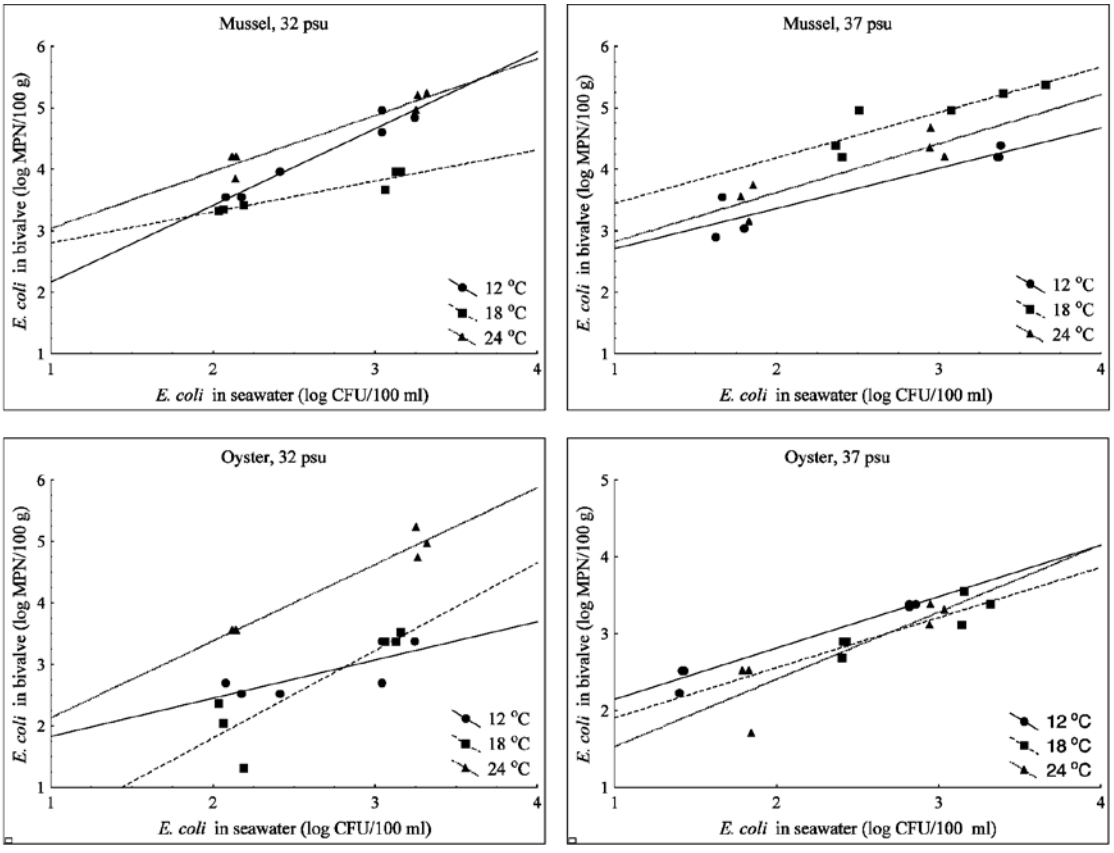

Fig. 3. The impact of salinity and temperature on the relationship between the concentration of E. coli *in bivalves and in seawater*

correlation was positive. The highest CR values at a salinity of 37 psu were found at 12°C, while at a salinity of 32 psu the highest CR was found at 24°C (Fig. 4).

These results are consistent with the results of previous studies (BERNARD, 1989) where the highest accumulation of coliform bacteria was recorded at 17°C in mussels (*Mytilus edulis*) and at 12°C in oysters (*Crassostrea gigas*). The highest filtration rate in mussels (*Mytilus edulis*) at a salinity of 38 psu occurred at the optimum temperature, which ranges from 15°C to 25°C without any definite peak (SHULTE, 1975). The highest filtration rates occurred at the optimum temperature, and decreased filtration rates at higher and lower temperatures were also found by ALI (1970), LAING (2004) and ŠOLIĆ *et al.* (2007). In experiments investigating the removal of a neutral red solution, COLE & HEPPER (1954) showed that the filtration rate in mussels (*Mytilus edulis*) was also a function of salinity. In the range of temperature from 1-11°C and salinity from 23.5-30.5 psu, they recorded increased filtration rates at higher salinities. RAJESH *et al.* (2001) found a positive correlation between salinity in the range of 10-32 psu and filtration rate in blue mussels (*Perna viridis*), while in oysters (*Crassostrea madrasensis*) the correlation was negative.

The relationship between the CR in mussels and oysters (CR_{Mussel}/CR_{Oyster}) was in the range of 7.7-38.5, and indicated significantly higher accumulation of *Escherichia coli* in mussels than in oysters under all experimental conditions (Fig. 5). At lower salinity (32 psu), the ratios were inversely proportional to temperature, while at a higher salinity (37 psu), the highest ratio was found at 18°C.

In addition to specific metabolic activities as a result of variations in temperature and salinity, the differences in the CR in mussels and oysters could also be attributed to different efficiencies of particle retention, which is a function of parti-

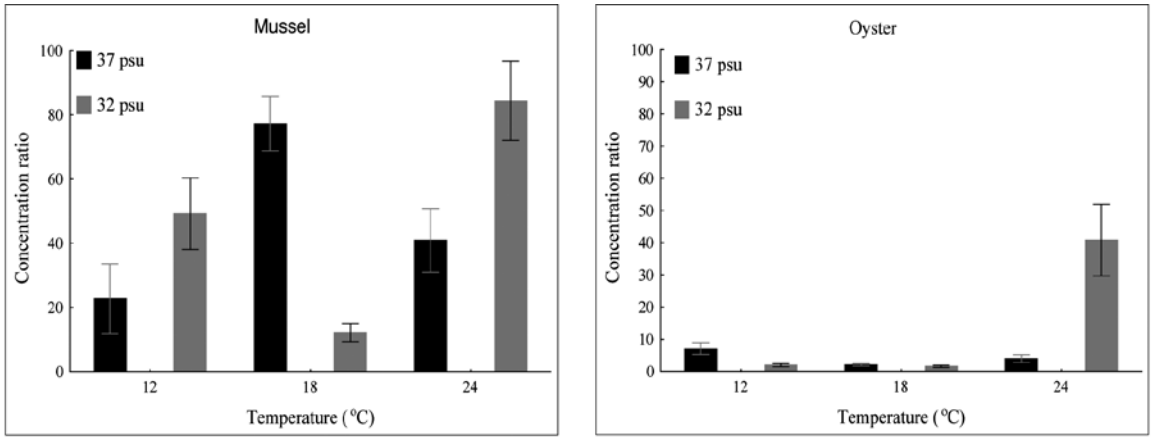

Fig. 4. Concentration ratio (CR) in mussels and oysters as a function of temperature and salinity (mean values ± SD)

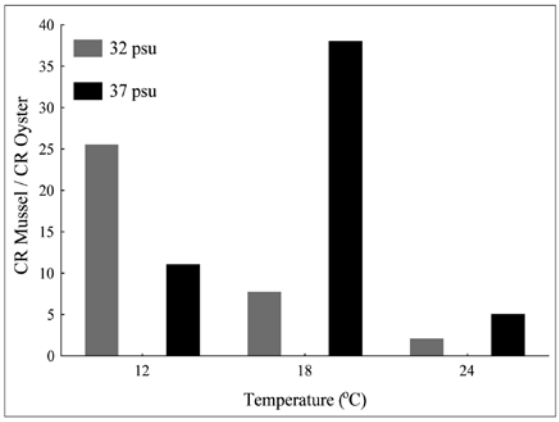

Fig. 5. Ratio between the concentration ratio (CR) in mussels and oysters (CRMussels/CROysters) as a function of temperature and salinity

cle size. According to MØHLENBERG & RIISGÅRD (1978) and SILVERMAN *et al.* (1999), the efficiency of particle retention in bivalves is most likely determined by the structure of the latero-frontal cirri. Each latero-frontal cirrus consists of a double row of 20-25 pairs of smaller cilia that beat in such a way as to form a meshwork between the cirri and adjacent filaments; this acts as a filter which should be capable of retaining particles from water passing through the gills. Mussels possess large, complex and well-developed latero-frontal cirri composed of 18-26 pairs of cilia (SILVERMAN *et al.,* 1999), and their efficiency in particle retention is significantly higher than in oysters which possess short latero-frontal cirri (JORGENSEN, 1990). In this way, mussels are able to retain 90% of particles up to 3 μm and 50% of particles up to 1 μm, while the same retention efficiency in oysters is achieved with much larger particles, 4.5 μm and 2 μm, respectively (JØRGENSEN, 1990).

CONCLUSIONS

This study clearly showed that in the studied range of *E. coli* concentrations in seawater, the *E. coli* concentrations in mussels and oysters exceeded the concentrations in seawater by an order of magnitude at least, and reached maximum values (plateau) in the initial phase of accumulation. Under the same conditions, mussels reached the plateau earlier than oysters and exceeded the concentrations in oysters by an order of magnitude. The level-off concentrations of *E. coli* in bivalves showed a significant positive correlation with their concentrations in the surrounding seawater, while the ratio between these concentrations (CR; concentration ratio) was controlled by temperature and salinity. In mussels, variations in temperature had a significant effect on CR, while variations in salinity had a significant effect only in the interaction with temperature. In oysters, variations in temperature had a more significant effect than variations in salinity. The statistically significant interaction between temperature and salinity suggested that, in both bivalve species, the simultaneous effect of temperature and salinity on CR was significantly greater than when each of these factors acted independently. At higher

salinity (37 psu) levels, mussels reached the highest *E. coli* concentrations at a temperature of 18°C, and oysters reached a plateau at 12°C. At lower salinity (32 psu) levels, both bivalve species achieved the highest *E. coli* concentrations and the highest CR values at 24°C.

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Akumulacija indikatorske bakterije *Escherichia coli* u dagnji (*Mytilus galloprovincialis*) i kamenici (*Ostrea edulis*) u eksperimentalnim uvjetima

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SAŽETAK

U ovom radu istraživan je zajednički utjecaj temperature i saliniteta morske vode na akumuliranje bakterije *Escherichia coli* u dagnji (*Mytilus galloprovincialis*) i kamenici (*Ostrea edulis*) u eksperimentalnim uvjetima pri različitim koncentracijama *E. coli* u morskoj vodi. Eksperimenti su provođeni u bazenima volumena 3000 L, u prirodnim rasponima temperatura (12°C, 18°C i 24°C) i saliniteta (32 psu i 37 psu), karakterističnim za priobalje Jadranskog mora. U istraživanom rasponu koncentracija *E. coli* u morskoj vodi (4x10-4.6x10^3 EC/100 ml), zabilježena je brza akumulacija bakterija u školjkašima, a najviše koncentracije (prag) dosezane su u prvom satu kod dagnje i drugom satu kod kamenice. Pri jednakim koncentracijama *E. coli* u morskoj vodi više maksimalne koncentracije *E. coli* zabilježene su kod dagnje. Utvrđena je jasna pozitivna povezanost između koncentracije *E. coli* u ambijentalnoj vodi i njene koncentracije u školjkašima (CR;concentration ratio-omjer koncentracija). Utvrđeno je također da je CR kontroliran temperaturom i salinitetom morske vode. Kod dagnje su promjene temperature značajno utjecale na promjene CR, dok su kod kamenice, osim temperature, na promjene CR značajno utjecale i promjene saliniteta. I kod dagnje i kod kamenice, zajednički učinak temperature i saliniteta bio je značajniji od njihovog pojedinačnog učinka. Najviše koncentracije *E. coli* u školjkašima za najmanje jedan red veličine su premašivale koncentracije u moru, dok je omjer najviših koncentracija u dagnjama i kamenicama bio u rasponu 7.7- 38.5.

Ključne riječi: *Mytilus galloprvincialis*, *Ostrea edulis*, *Escherichia coli*, omjer koncentracija, temperatura, salinitet