

FAECAL CONTAMINATION OF MYTILUS GALLOPROVINCIALIS CULTIVATED AT BUTRINTI LAGOON AND ANNUAL AREA CLASSIFICATION

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Abstract

Fecal coliform bacteria are indicator organisms used to determine the presence of sewage or fecal matter and serve as indicators of unsanitary conditions in the aquatic environment. Bivalve mollusks are filter-feeding organisms and when a shellfish bed is contaminated by fecal bacteria, the molluscs absorb and concentrate these contaminants. Coliform bacteria have no effect on shellfish survival or growth, but they can render them unfit for human consumption particularly when consumed raw or lightly cooked. Butrinti lagoon and Shengjini bay are favorable habitats and the main areas in Albania, where the *Mytilus galloprovincialis* is cultivated. The first objectives of this paper is to examine the level of fecal indicator *Escherichia coli*, the *Salmonella* spp. presence/absence, in cultivated mussel of Butrinti lagoon. The annual area classification, based on *Escherichia coli* level is the second objective. 81 samples, collected biweekly in three Butinti lagoon stations, during 2012, were tested for *Salmonella* presence, *Escherichia coli* enumeration with the methods recommended by EU. Only 64.2% of samples fulfilled the veterinary conditions about placing live shellfish on the market, concerning the level of 230 E. coli in 100 g of flesh. Meanwhile, 27.2% belonged Class B, where mollusks must be purified. 8.6% belonged to Class C, where bivalves must be relayed 2 months to meet Class A or B. *Salmonella* spp. was detected in one case out of 81 samples. The contamination level were almost similar between three sampling stations. The fecal contamination and area classification was closely related with seasons. During spring-summer 100% of the samples were classified in A class, while B and C classes appeared during fall-winter.

Keywords: *fecal contamination, Mytilus galloprovincialis, Escherichia coli, Salmonella spp., area classification, Butrinti lagoon.*

Introduction

Albanian shellfish farming is concentrated in the Butrinti lagoon although some activity is exercised also in Lezha, Shengjini bay. Mussel culture in Albania has been developed since the beginning of the '60 in the coastal lagoon of Butrinti, located in the Ionian Sea (Fig.1) covers an area of 1600 ha, with a depth of 20 m and communicates with the sea by a 3,8 km long canal. This lagoon is artificially filled with freshwater, so the salinity ranges from 18 to

30%. (11). The fixed structures (80 units) have been used for the production of the mussels (*Mytilus galloprovincialis*). The production of the mussels was growing up year by year, up to a maximum of 5000 tons/year in the last of '80 (19). After 1995, mussel breeding was practically stopped, both for internal organizational reasons, but above all because of the block on exports imposed by the EC for sanitary reasons, in October 1994. Until now are under production about 59 equipments (fixed concrete units), by total production of 1200 Ton, also by floating units with 32ha , 2000 tons (20).

The water temperature less than 10 degrees during the winter, and about 27 degrees C in summer, is an optimal condition for cultivation of *M. galloprovincialis*.

Bivalve mollusks are filter-feeding organisms and when a shellfish bed is contaminated by fecal bacteria, the mollusks absorb and concentrate these contaminants. (5,7). Coli form bacteria have no effect on shellfish survival or growth, but they can render them unfit for human consumption particularly when consumed raw or lightly cooked (14).

Microbiological control of bivalve mollusks and their habitat is considered an important process for their safety, and is the obligation for competent authority, derived from directive of European Community, Directive 91/492. The level of fecal indicator, such as *Escherichia coli* in bivalve mollusks shows how much fecal pollution they have been exposed to in the harvesting area and determines what, if any, treatment shellfish require before they are consumed. Based on the levels of *Escherichia coli* it is performed the classification of production areas.

The requirements for the production, harvesting, storage conditions, and use in food processing industry of bivalve shellfish are described in the EU food hygiene package (16, 17, 18). According to the European Community under Regulation 854/2004, the production areas are classified into categories:

Table No.1: The classification of production areas

Classification of production areas	Microbiological standards for 100g of meat and liquor	Treatment required
A	≤ 230 E. coli/100g	None, direct for human consumption
B	230 - 4 600 E. coli/100g	Must be depurated, heat treated or relayed to meet class A requirements
C	4 600 - 46 000 E. coli/100g	Must be relayed for 2 months to meet class A or class B requirements

Materials and Methods

Mussel culture was practiced mainly in the raft system suspended culture in the Butrinti lagoon. Bivalve molluscs are monitored from designated sampling sites within production areas according to a sampling programme set by CA (competent authority, MAFCP). Currently, classification requires a minimum 6 samples to be tested in separate months over the year for a given area, although in the majority of cases monitoring is conducted on additional months throughout the year. For the purpose of this study 81 samples of mussel *Mytilus galloprovincialis*, was conducted during 2012, in three stations of the Butrinti lagoon: North, West and South, with a biweekly sampling frequency. All samples were tested for *E. coli* level and *Salmonella* spp. Within Europe, ISO TS 16649-3:2005 (based on Donovan et

al. 1988) is the reference method for the testing of shellfish for *E. coli*. This method is a two-stage; five tube by three dilution MPN. The first stage of the method is a resuscitation requiring inoculation of minerals modified glutamate broth (MMGB) with a series of diluted shellfish homogenates and incubation at 37 ± 1 degrees C for 24 ± 2 hours. The presence of *E. coli* is subsequently confirmed by subculturing acid producing tubes onto agar containing 5-bromo-4-chloro-3-indoly- β -D glucuronide (TBX), incubated in 44 ± 1 degrees C for 21 ± 3 h, and detecting growth on the tryptone bile glucuronide agar (TBX). The presence of green to blue colonies as a result of the action of β -glucuronidase enzyme on the TBX medium indicates the presence of *Escherichia coli* in MMGM tubes. The Most Probable Number (MPN) acquired is calculated on the basis of a chart of the methodology presented in ISO 7218:2007, considering the positive tubes for each dilution.

Detection of *Salmonella* spp. The presence of salmonellas in shellfish was determined in accordance with the recommended EU method, i.e. in line with the ISO 6579 standard, an accredited method. The presence of salmonellas was determined in 25 g of whole flesh. The prepared samples were pre-enriched by means of 9-times the amount of peptone water and incubated for 16 - 20 h at 37 °C. Afterwards, the samples were subcultured to two liquid enrichment media: Rappaport – Vassiliadis soya broth or RVS and Mueller Kaufman tetratonat with novobiocin (MKTTn).The first medium was incubated at 37 °C, and the second at 41.5 °C for 24 h. From the enrichment media samples were subcultured to the selective media xylose lysine desoxycholate agar XLD and Hectoen agar. The plates were incubated for 24 h at 37 °C. After incubation, the plates were examined for the presence of any characteristic colonies. If they were found, the API-20 E system it is used for the biochemical confirmations followed by serological test.

Results and Discussion

The results of the examinations perform to the 81 mussel samples collected to the raft system suspended culture, are shown in the chart (Chart No.1) and the table 1. From results obtained during 2012, the levels of contamination with *E.coli* varies from <20 till 16000 MPN *E.coli*/100g.

Chart No.1: Distribution values of E.coli log MPN/100g for 81 samples of live bivalve mollusk

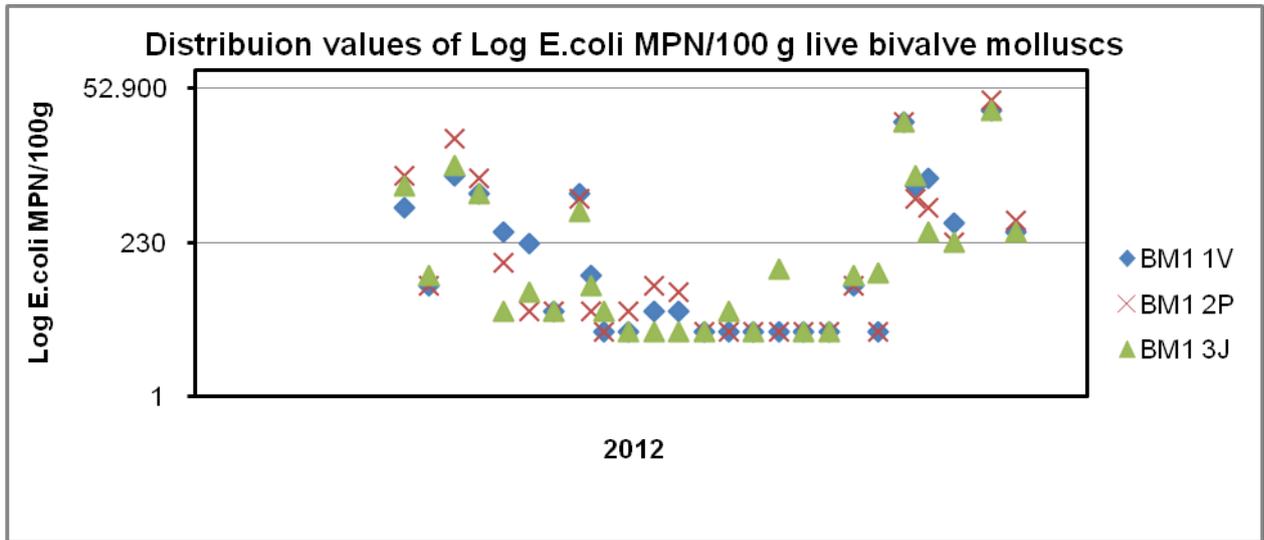
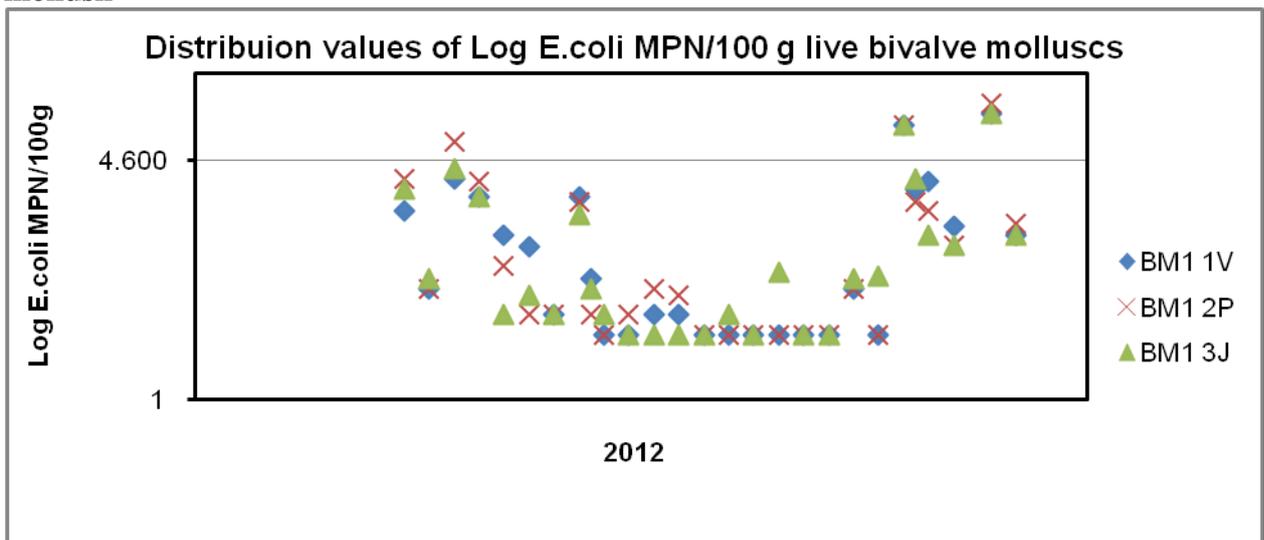


Chart No.2: Distribution values of E.coli log MPN/100g for 81 samples of live bivalve mollusk



The number of E. coli determined in samples varied between <20 MPN/100 g till 16000 MPN/100 g (MPN, most probable number) and the majority of them (64.2.0%) contained E. coli in quantities not exceeding the acceptable limit of 230 MPN/100 g. However, E. coli was present in number higher than 230 MPN/100 g in (35.8%) samples, also higher than 4600 MPN/100 g in (8.6%) of samples. Escherichia coli in particular is now considered the most reliable indicator of the microbiological quality of coastal waters. It represents the dominant faecal coliform in human and animal faeces and is the most stable in aquatic environments (3). The quantification of Escherichia coli is, therefore, much more important than that of faecal coliforms in the evaluation of water quality (4).

The classifications are based on the EU above described criteria. The results of 81 samples analyzed during 2012, showed that 64.2%, of the samples fulfill the requirement of being

class A area, meanwhile 27.2% of the samples B area, and 8.6% of the samples C area. These results of the 2012 year classification are presented in the chart below:

Chart 3: Number of samples for each of the classification band (year 2012)

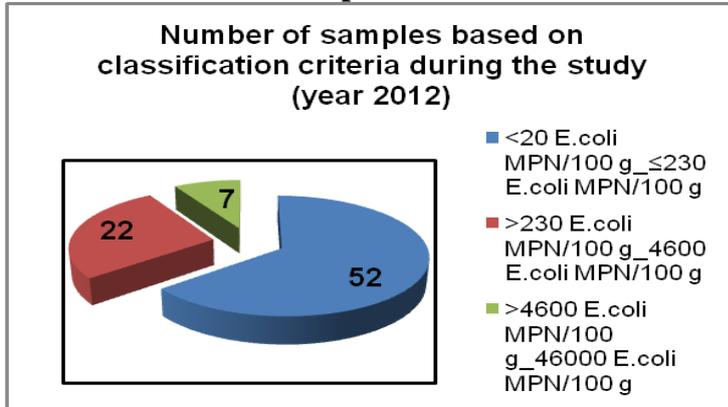
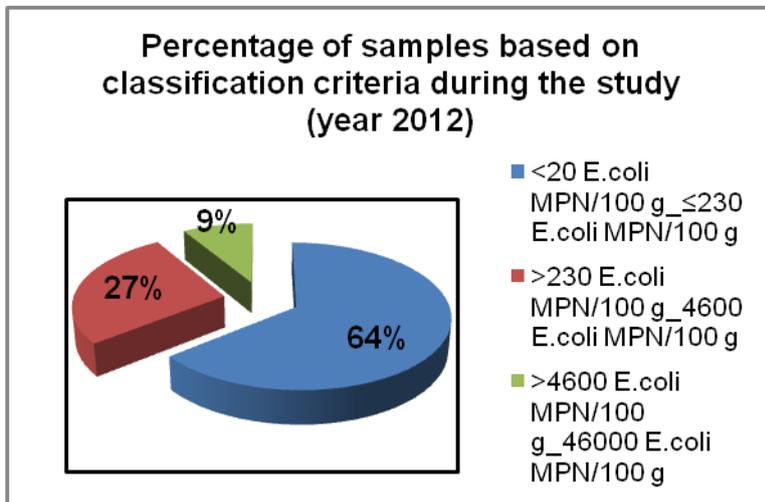


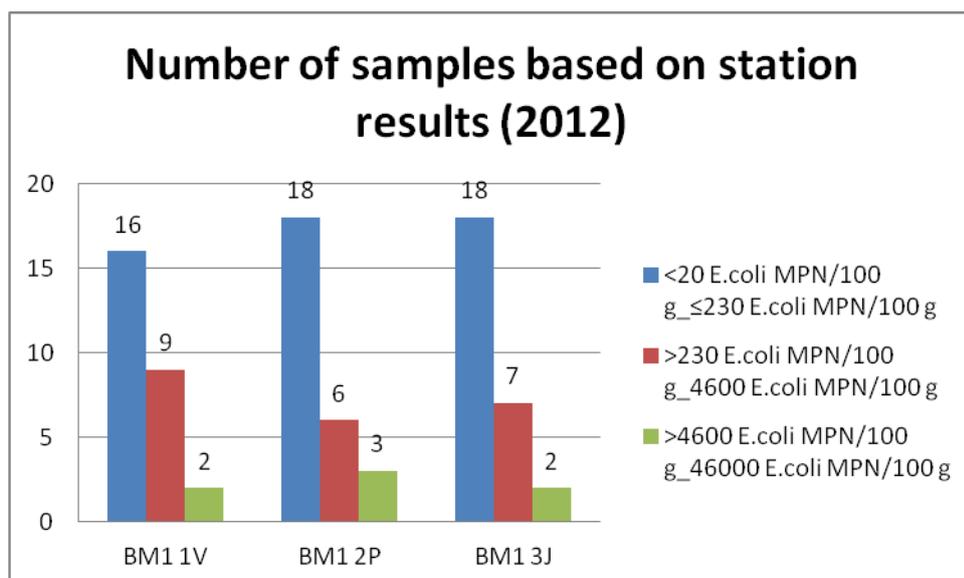
Chart 4: Percentage of samples for each of the classification band (year 2012)



Based on the results expressed by chart 4, compare to the established criteria for the production areas, shellfish produced from the Butrinti lagoon area monitored fall into Class B which indicates that they can not be placed on the market for direct human consumption and can only be placed in the market after depuration or relaying. The same classification status was given to this area from the beginning of the monitoring process and the classification is sustainable (7,8).

The result of the exanimate mussel samples collected to three sampling stations are showed in the chart 5.

Chart 5: Number of the samples for each station, belong to the classification band (year 2012)



The number of samples contaminated with *E.coli*, for each classification band, and the contamination level situation are almost similar between three sampling stations of the lagoon. At the three stations most of the samples belong to the band < 230 *E.coli* MPN/100g, meanwhile is nearly the same number of samples belong to two other bands.

The three stations of this harvesting areas are classified as Class B which means that producers have to purify (i.e. hold in depuration units) their bivalve mollusks in a depuration unit before selling them into the food supply chain.

To protect human health, all mollusk shellfish must meet an end product standard of less than 230 *E coli* per 100g of flesh before they can be placed on the market.

Indeed, animals that have been through an approved depuration unit that is operated to EU standards should achieve a microbiological level of 75 *E coli* or less (15)

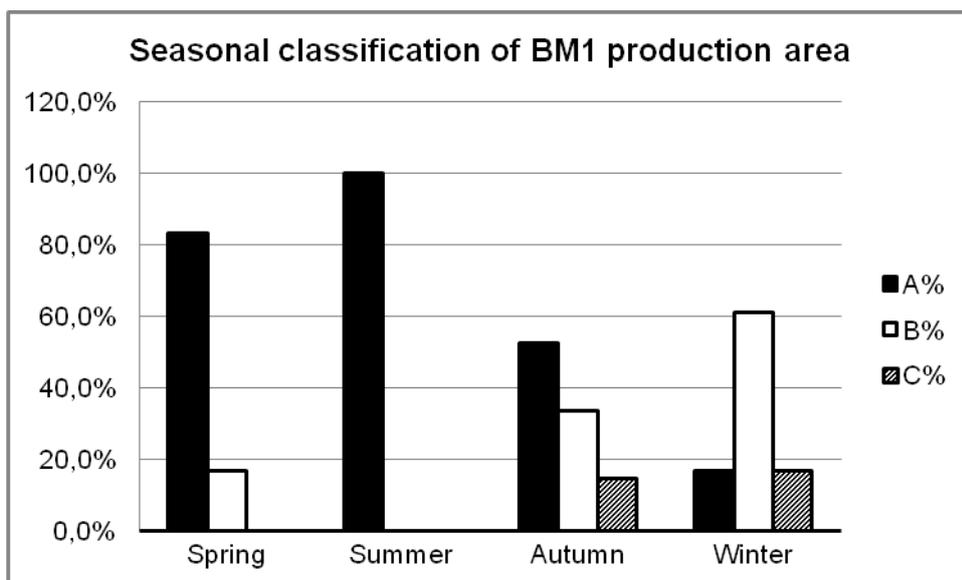
Related to the seasonality, it is clearly visible from Table No 1.,2 that *Escherichia coli* values during summer are 100% A area. Meanwhile B area appears in spring and autumn and the situation deteriorates further in the winter season with the appearance of C area with an very high season mean value of *E.coli* arrives to 8223, and an equal percentage between B and C area (16.7 %).

Table No 1: *Escherichia coli* mean values for each season

Season	Means of season values year 2012		
	BM1 1V	BM1 2P	BM1 3J
Spring	248	169	109
Summer	12	15	25
Autumn	2919	2599	2731
Winter	4812	8223	5150

Table No 2: Percentage of samples for the classification band, for each season

Season	A	B	C
Spring	83,3%	16,7%	0,0%
Summer	100,0%	0,0%	0,0%
Autumn	52,4%	33,3%	14,3%
Winter	16,7%	61,1%	16,7%



Salmonella detection

In the study, *Salmonella* sp. was found in only one of the examined samples or 1.2 %. Other investigations concerning live bivalve molluscs confirmed that *Salmonella* sp. can tolerate coastal water salinity. The bacteria may be found in 0%-2.5% of live shellfish and consumption of these foods may lead to salmonellosis, characterized by enteric fever along with gastroenteritis and diarrhoea (12, 15). The presence of *Salmonella* sp. in water depends on many factors, including human and animal fecal pollutions (14). However, epidemiological data indicate that prevalence of *Salmonella* strains in mollusks may vary from 3% to even 34% in areas with warm sea water such as Vietnam or India (12). In Europe, according to the data published by EFSA, *Salmonella* sp. remains the main causative agent, responsible for 30.5% of noted outbreaks associated with the consumption of food, where shellfish and mollusks were the source in 6.8% (13).

Conclusions

- The study showed that the cultivated mussel of Butrinti lagoon are contaminated with *E.coli*, and the level varies from < 20 till 16000 MPN 100g flesh end liquid intravalular
- Butrinti lagoon production area during 2012, based on *Escherichia coli* level results compare to legislation criteria, is classified as an B area, because only 64.2% of the samples has the level under or 230 MPN/100g.

- Based on the results of the stations, is evident the fact that no differences in contamination level and classification between the three stations.
- There were differences in the E.coli level and the classification between the seasons. In this study we see that in Summer the mean value of E coli for three stations is very low (12, 15,25) and the classification of the production area is 100% A area, meanwhile B and C area appears during Spring, Autumn and Winter.
- Referring to our results and those of the previous years, the levels of E.coli varies, but the classification of production areas is sustainable (B area).
- Classification of shellfish in the production area B and C means that they must necessarily pass the depuration process, to minimize the values of *Escherichia coli*, before placing on the market.
- There are some factors which play an important role in the *Escherichia coli* contamination, of the Butrinti lagoon cultivated mussel such as municipal sewage and private activities, wastewater sources, rain precipitation and river flows from Bistrica.
- Safety of this product can be guaranteed mainly by preventive measures and application of appropriate procedures such as classification of harvesting areas, monitoring programs, control of depuration process, final product inspections, and hygiene control for food business operators involved in whole food chain.
- The consumers should be provided with a clear information on possible health hazards associated with the consumption of these products.

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