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Depuration of Striped Venus Clam (*Chamelea gallina* L.): Effects on Microorganisms, Sand Content, and Mortality

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ABSTRACT: This study was focused on the evaluation of the microbiological indices, defined by European legislation, before and after the depuration of clams (*Chamelea gallina*) landed in category B seawater. The survival of depurated clams and the meat yield were also evaluated. The results obtained from October 2002 to September 2003 evidenced a mean microbial reduction during depuration of 62% for *Escherichia coli* and 54% for fecal coliforms (FC). All the samples had FC counts below the limit after 24 h depuration with the exception of the August samples. *E. coli* was found in concentration slightly higher than the legal limit only in the samples of December and January. In August, the *E. coli* concentration did not decrease during the depuration, while in the other samples significant reduction of *E. coli* concentration was observed. *Salmonella* spp. and *V. parahaemolyticus* were never detected in the clams harvested between March and September. *Vibrio alginolyticus* was found in the clams harvested in May and September both before and after the depuration process. The viability of clam was not negatively affected by depuration, in fact, an increase of viability was observed with the exception of the samples of April. The meat yield was not influenced by the depurative treatment in *C. gallina*; the mean value found before depuration, 10.47% (with 1.95 SD), did not significantly vary after the treatment (10.58%, SD 2.07). In conclusion, the depuration conditions can improve the quality of *C. gallina*; however, its effects on microbiological quality depended on environmental conditions.

Keywords: *Chamelea gallina*, depuration, microbiological indices, quality, shellfish

Introduction

The venerid bivalve *Chamelea gallina* (striped venus or baby clam) is a mollusc distributed throughout the Mediterranean and Black Sea (Moschino and Marin 2006). It can be found in sea bottoms of clean and muddy sand from the lower shore to depths of approximately 15 m (Morello and others 2005). It is particularly diffused in the inshore waters of Italy, Spain, Turkey, and Morocco (Ramon and Richardson 1992). The landing of *C. gallina* has an important economic role in the central and northern Adriatic coasts of Italy where it has considerably increased in the last decades with the introduction of the hydraulic dredges (Morello and others 2005; Moschino and Marin 2006). The interest toward this clam increased recently also in relation to its nutritional characteristics. In fact, it has interesting dietetic properties due to the low lipid and cholesterol contents, to the presence of phytosterols and to the high percentages of healthy polyunsaturated fatty acids (Orban and others 2006).

Bivalve molluscs can accumulate microorganisms, including pathogens, from seawater (Cook 1991). In fact, they are filter-feeding organisms and can concentrate bacteria even in high number (Šolić and others 1999). The number and type of microorganisms present in the water depend on several seasonal, climatic, and anthropogenic factors. In Italy, the production, harvesting, and commercialization of bivalve molluscs are regulated by the European Community (EC). The EC directive 79/923 (Anonymous 1979) defines the classification of the waters in which the molluscs can be harvested, while the EC directive 91/942 (Anonymous 1991) states the safety standards for live mollusc sale. These indications have been detailed and integrated by EC regulations 466/2001 (Anonymous 2001) and 221/2002 (Anonymous 2002) and recently confirmed by EC regulations 2073/2005 (Anonymous 2005). In Italy, before the application of this latter regulation, the microbiological quality of shellfish was based on legal requirements derived from an extensive interpretation of the previous EU indications. In fact, for the molluscs, in addition to the absence of *Salmonella*, a maximum tolerable cell concentration was required both for fecal coliforms (FC) (300 MPN/100 g meat) and *Escherichia coli* (230 MPN/100 g meat).

C. gallina is landed in natural beds usually located in areas classified, on the basis of the microbiological parameters, as category A by EC Directive 91/492 (Anonymous 1991) and it can be marketed without any preventive treatment.

The often-uncontrolled fishing of the last years, coupled with some recent uncommon meteorological events, has drastically reduced the clam biomass in the Adriatic Sea (Moschino and Marin 2006). Moreover, it has been observed that often commercial samples of *C. gallina* do not meet the microbiological limits required by European directives (Gardini and others 2000). In this framework, also clams landed in beds located in water classified as category B could be an important source for the local fish industry. However, such clams need a depuration step able to reduce the FC and *E. coli* population below the limits requested for marketing.

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Depuration is a process in which the shellfishes are placed in tanks with clean seawater and allowed to resume their natural filter-feeding activity purging themselves of sewage contaminants. This process was initially developed in response to a number of outbreaks of shellfish-associated typhoid illness (Roderick and Schneider 1994). Several factors influence the degree of depuration. These include system design, initial water quality, oxygenation and flow rates, salinity, temperature, shellfish-to-water ratios, removal and settlement of fecal material, and the period of purification (Lee and Younger 2002). The conditions for a satisfactory depuration have been defined for several bivalve mollusc species, including oysters (*Ostrea edulis, Crassostrea gigas*), mussels (*Mytilus edulis*), cockles (*Cardium edule*), great scallops (*Pecten maximus*) and many clams (*Ensis* spp., *Mercenaria mercenaria*, *Tapes philippinarum*, *T. decussatus*, and *Spisula solida*) (Doré and others 2003). No information is reported for *C. gallina* depuration. This is probably due to the fact that its production from water classified as A had been adequate to the demand until some years ago. Moreover, the operators often expressed the belief that depuration had a negative impact on the survival of *C. gallina* during storage and marketing.

The aim of this study was to evaluate the behavior of the microbiological indices defined by European legislation during the depuration of clams belonging to the species *C. gallina* landed in category B seawater, and verify the survival of the clams after depuration, during a storage carried out at market conditions. The depurative protocol adopted was the same commercially used for the depuration of the Manila clam (*Tapes philippinarum*).

Materials and Methods

Collection of samples

The depuration trials were carried out for 1 y, from October 2002 to September 2003 with monthly treatments. The *C. gallina* clams were harvested from offshore natural beds located in the Adriatic Sea adjacent to Cattolica (Italy). After landing, each sample (about 40 kg) was immediately refrigerated (4 °C) and transported to the depuration plant. About 10 kg of clams were used for the detection of FC and *E. coli* (time 0), and the remaining were subjected to depuration and microbiological analyses were performed after 6, 9, and 24 h.

Clam depuration

Depuration was conducted in 15000-L commercial baths (length of bath, 9.8 m; width, 1.3 m; depth of water, 1.2 m) with an open-circuit seawater-disinfection system that uses both filtration and ozone, commercially used at the Bivalve Depuration Center (Cattolica, Rimini, Italy). The seawater flowed at a rate of about 18 L/min and a complete water cycle needed about 14 h. The seawater was taken from open sea and its parameters depended on the season. Table 1 reports the salinity, temperature, dissolved oxygen, oxygen saturation, and pH for each depuration cycle carried out in this experimentation. The clams were elevated from the bottom of the bath on a perforated plastic tray to curtail recontamination with feces and pseudofeces. Each plastic tray contained about 8 kg of clams with a density of about 29 kg/m^2. Clams (about 3 kg/sample) were removed at 0, 6, 9, and 24 h for analyses.

Microbiological analyses

For each sampling time microbiological analyses were performed in triplicate, in particular 3 subsamples (each of about 1 kg) were used. The clams were scrubbed free of dirt, washed in hypochlorite solution (20 mg/L), rinsed with sterile distilled water, and shucked with a sterile knife. Tissue and shell liquor samples (about 100 g) were homogenized in a Stomacher (BagMixer, Interscience, France).

FC were enumerated through a 5 tubes per dilution most probable number (MPN) series (Hunt and Springer 1978). The tubes contained 9 mL of Bacto A-1 Medium (Difco, Detroit, Mich., U.S.A.). After 3 h at 37 °C plus 21 h at 44 °C, gas positive tubes were recorded for FC. From each FC gas positive tubes, 0.1 mL was transferred in tubes with 10 mL of Tryptone Water (Oxoid, Basingstoke, U.K.) and then incubated for 24 h at 44 °C. *E. coli* was enumerated by adding 0.5 mL of Kovac's indole reagent (Merck, Darmstadt, Germany) to each tube. After 10 min, tubes with a dark red color in the alcohol layer were recorded for *E. coli*. For the evaluation of the presence of *Salmonella* spp. in 25 g of mussels homogenates the method of the Intl. Organisation for Standardisation (ISO 6579, 1993) was followed.

Qualitative *Vibrio* spp. and *Aeromonas hydrophila* analyses were carried out on 25 g of wet meat which were added to 225 mL of 3% NaCl containing phosphate buffered peptone water (peptone 10 g, sodium phosphate dibasic 3.5 g, potassium phosphate monobasic 1.5 g; final pH 7.2 ± 0.2), homogenized and incubated for 24 h at 37 °C. After this period, a loopful of homogenate was streaked onto Thiosulphate Citrate Bile Salt (TCBS, Oxoid) and incubated for 24 h at 37 °C. The suspected colony types (*Vibrio* colonies: yellow–greenish-blue; *Aeromonas* colonies: yellow) were picked out, streaked on to TCBS and incubated at 37 °C for 24 h. The isolates were further characterized using API

Table 1 — Physicochemical characteristics (salinity, temperature, dissolved oxygen, pH) and changes in *E. coli* and fecal coliform counts (%) for each depuration cycle during October 2002 to September 2003. The variation of microbial counts represents their percentage reduction after depuration treatment. Negative values indicate an increase of the counts after depuration.

Sample	Salinity (mg/kg)	Temperature (°C)	Dissolved oxygen (mg/L)	pH	Variation in *E. coli* counts (%)	Variation in fecal coliform counts (%)
Oct 2002	33	17.8	5.82	8.45	98.0	99.1
Nov 2002	36	16.4	6.14	7.72	100.0	98.7
Dec 2002	22	9.9	4.93	7.86	67.3	67.3
Jan 2003	30	7.0	5.85	8.05	60.7	47.6
Feb 2003	35	6.9	9.11	7.99	100.0	24.8
Mar 2003	33	10.4	7.71	7.90	0.0	0.0
Apr 2003	35	12.4	3.05	8.00	98.0	30.3
May 2003	35	18.7	4.53	7.73	43.5	43.5
Jun 2003	34	25.2	3.27	7.98	–5.4	–19.6
Jul 2003	37	24.9	5.07	7.45	100.0	100.0
Aug 2003	36	25.4	6.42	7.96	–3.0	–43.5
Sep 2003	36	20.6	5.07	7.92	100.0	100.0

20 NE and API 20 E tests (Biomérieux, Marcy l'Etoile, France) to confirm the species (Colakoglu and others 2006).

For the detection of *Listeria* spp. 25 g of sample were added to 225 mL Listeria Enrichment Broth Base (UVM) with Listeria Primary Selective Enrichment Supplement (UVMI) for 24 h at 32 °C, followed by the inoculum of 0.1 mL of enrichment into 10 mL of buffered Listeria Fraser Broth Base (Oxoid) incubated for 24 h at 32 °C. The positive tubes were streaked onto ALOA Agar Base (Oxoid) for 24 to 48 h at 35 °C. Typical colonies were selected and streaked onto Tryptone Soya Yeast Glucose Agar (TSYGA) or Listeria Selective Agar Base (Oxoid). The isolates were assigned to the species according to API Listeria (Biomérieux).

Yield, sand content, and mortality of clams

The yield, that is, the percentage ratio between meat content and total weight of clams, was determined in triplicate by boiling 3 subsamples of clams (each of about 0.8 kg) for 4 to 6 min in a stainless steel vessel with 750 mL of water; after cooling, the meat was separated from the shell, drained, and weighted.

For the ash (including sand) determination, 3 subsamples of clams (each of about 0.8 kg), sized between 22 and 33 mm, were rinsed under running water and then put in a steel vessel with 700 mL of distilled boiling water, until valves opened. After cooling, each sample was washed, the intervalval liquid was separated from flesh and filtered on to 2 nylon sieve, the first with 1 mm meshes and the 2nd one with 36 μm meshes. The filtered intervalvar liquid was placed in a porcelain crucible and put in a stove for 24 h at 105 °C until constant weight; after it was introduced in a muffle to reduce to ashes for 4 to 6 h at 600 °C until constant weight. The tare of the crucible was measured before each analysis after a treatment for 15 min at 105 °C.

Mortality of clams was measured in samples of about 2 kg harvested from March 2003 to September 2003. For each sampling time clams not depurated and stored at 4 to 6 °C were considered as control. The depurated samples were stored at the same temperature immediately after depuration. From each sample, empty, broken, and dead clams; valves with fractures; and clams with open valves that did not respond to the stress solicitation were preventively eliminated. The remaining viable clams of each month (both depurated and control) were counted before refrigeration. The mortality was determined by visual and tactile analysis of each clam and was performed daily over a period of 8 d. The mortality was expressed as percentage of dead clams per day.

Statistical analyses

Standard deviations of the 3 replicates for each sample are reported as bars in the figures. The *t*-test was performed using the statistical package Statistica for Windows 6.1 (Statsoft Inc., Tulsa, Okla., U.S.A.).

Results

Microbiological analyses

The presence of FC and *E. coli* in *C. gallina* before and after 24 h of depuration is reported in Figure 1 and 2. Table 1 reports the percentage reduction of the microbiological indices caused by the treatment (values with the negative sign indicate an increase of the microbial counts after depuration). The samples of January, April, and July had a FC concentration (Figure 1A) higher than that admitted by Italian regulation (300 cell/100 g of wet meat) but only the samples of July markedly exceeded this limit (700 cell/100 g of wet meat). All the samples had FC counts below the limit after a 24-h depuration with the exception of the August sample, in which the FC concentration slightly increased (up to 330 cell/100 g) during the 24-h depuration. In this sample, in fact, an increase in the FC concentration was observed during the first 9 h of depuration (as shown in Figure 1B). For other samples, such as June, no significant variation in the FC number was found throughout all the depurative cycle, while the samples of February, March, and April were characterized by reduced decreases in FC concentration (see the data of April in Figure 1B). On the other hand, the samples of October (reported in Figure 1B), November, December, and September showed a rapid and consistent reduction of the counts of this microbial group during depuration.

E. coli was found in concentration slightly higher than the legal limit (230 cell/100 g of wet meat) only in the samples of December and January, while in the samples of May, July, and August the counts coincided with this limit (Figure 2A). In the latter samples, the *E. coli* concentration did not decrease during any step of depuration as reported in Table 1 (the behavior observed in August is reported in Figure 2B). On the contrary, in the other samples, significant reductions of the concentration of this species were observed during depuration, as shown by the *E. coli* counts in April throughout all the depuration cycle (Figure 2B, Table 1).

The qualitative presence of some pathogens or potentially hazardous microorganisms was also monitored in the samples harvested between March and September. *Salmonella* spp. and *V. parahaemolyticus* were never detected in the clams analyzed. *Vibrio alginolyticus* was found in the clams harvested in May and September. In these samples, the presence of this *Vibrio* species was not eliminated by the depuration process adopted. In addition, in the sample of May the presence of *Aeromonas hydrophila*, *L. grayi*, and *L. ivanovii* (but not *L. monocytogenes*) was detected. However, after the depuration, none of these species was found in the samples landed in this month. In contrast, the presence of *L. grayi* was observed in the clams harvested in July before and after the depuration.

Yield, sand content, and mortality

Other analyses were carried out on the samples landed between March and September to evaluate the effect of the depuration on characteristics of the marketed clams. The meat yield was not influenced by the depurative treatment in *C. gallina*; the mean value found before depuration, 10.47% (with 1.95 SD), did not significantly vary after the treatment (10.58%, SD 2.07). Because of the characteristics of the seawater in which it grows, *C. gallina* had a naturally low sand content. Nevertheless, the ash content, which includes sand, of this clam decreased from 0.09 g/1000 g of meat (SD 0.04) to 0.02 g/1000 g of meat (SD 0.01) during depuration indicating a loss of sand.

Finally, the mortality of clams during storage at 4 to 6 °C was considered. It was expressed as percentage of clams of the same sample which lost their viability during storage. The mortality of depurated clams was compared with that observed in clams of the same lot not subjected to depuration. Interestingly, no increase was observed in the mortality of *C. gallina* due to the application of the depurative treatment (Figure 3). On the contrary, the mean mortality value observed between March and September 2003 after 5 d of storage, that is the usual time for commercialization assigned to these clams, was significantly lower in the depurated clams (3.83%) (Figure 3B) than in the not depurated ones (7.12%) (Figure 3A), as demonstrated by the P value associated to a t-test ($P = 0.0026$). In Figure 3 are reported the differences in mortality observed after 8 d of storage (33.71% in depurated and 39.55% in not depurated clams) even if in this case, the difference was less significant ($P = 0.0729$).

Discussion

In Italy, shellfish production reached a stable level at the end of the last decade. Actually about 180000 tons are produced, and the clams account for about 50000 tons (Saroglia and others 2000). Among clams, *C. gallina* has a significant economic relevance for the fishery of Adriatic Sea. However, landing activities have exposed *C. gallina* to overexploitation and its natural beds are now showing signs of depletion (Froglia 2000). In this framework, the landing of clams located in seawater classified as B can assume an economic importance, even if a depuration step has to be adopted before commercialization. Other clams (*Tapes philippinarum*) landed in the same area from category B seawater are currently depurated in plant in which natural seawater is used instead of artificial seawater. On the other hand, the use of artificial seawater for depuration has been demonstrated to be unacceptable for other molluscs (that is, scallops) because it increased remarkably the mortality of shellfishes (Doré and others 2003).

The mortality of clam was not affected negatively by depuration. On the contrary, a decrease of mortality was observed in all the depurated samples, except those of April. The mortality decrease after depuration was particularly relevant in the samples of June and August characterized by the highest mortality of untreated samples. The higher mortality of *C. gallina* observed in June and in August should be related to the higher temperature value

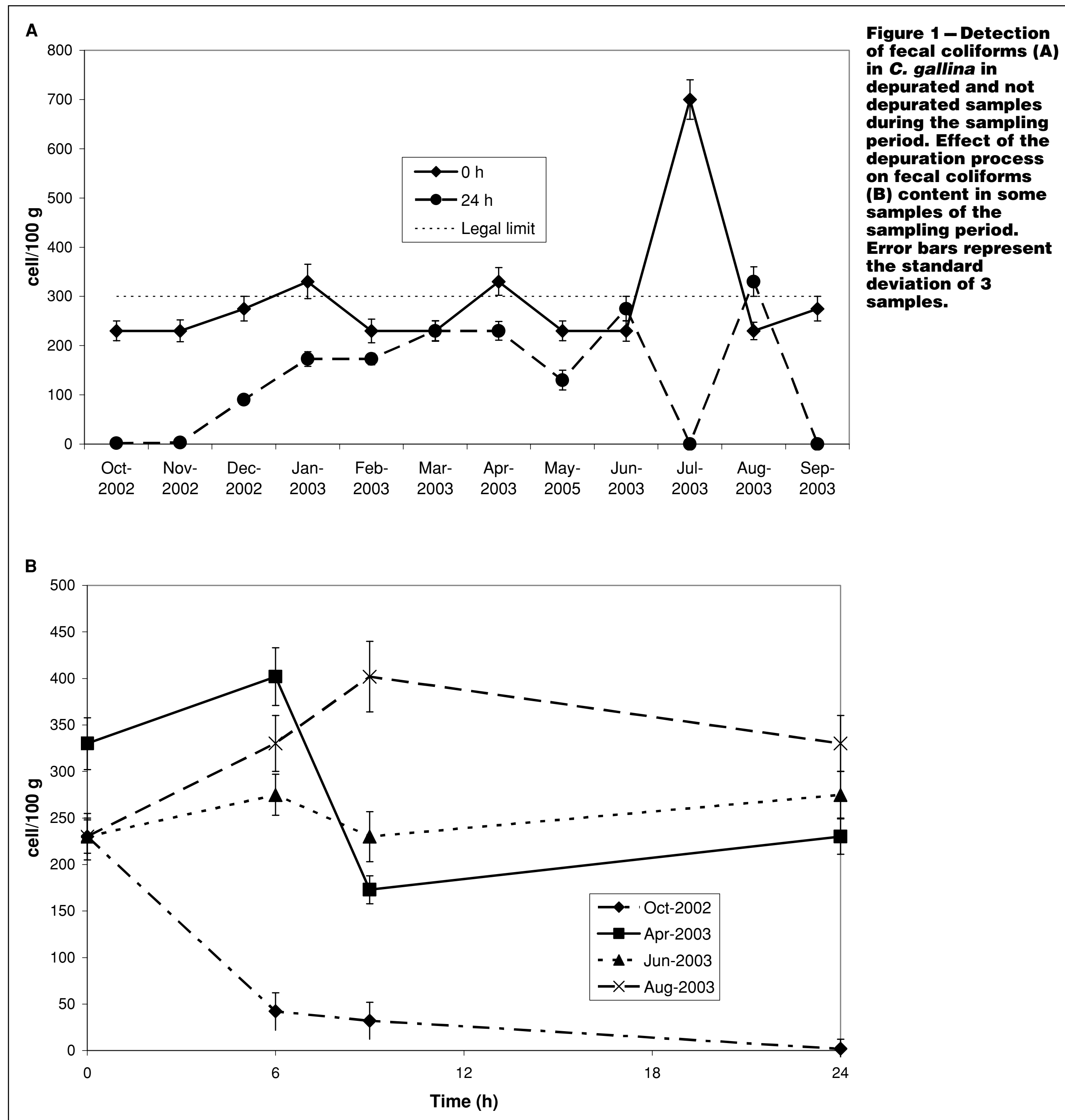

Figure 1 — Detection of fecal coliforms (A) in *C. gallina* in depurated and not depurated samples during the sampling period. Effect of the depuration process on fecal coliforms (B) content in some samples of the sampling period. Error bars represent the standard deviation of 3 samples.

of seawater used for depuration (25.2 and 25.4 °C, respectively), even if the samples of July did not show a mortality particularly enhanced in spite of a similar seawater temperature.

The clam samples studied in this experimentation were all landed from category B seawater. However, only 4 samples of *C. gallina* exceeded the microbiological limits before depuration and only one (August) did not comply with the legal limits after depuration. The anomalous FC peak observed in July was already observed in mussels landed in the same area in the same month (Vernocchi and others 2007). In that case, it was attributed to specific meteorological events that influenced the regimen of rivers and, consequently, the microbiological quality of seawater. Also for mussels the higher concentration of FC and *E. coli* was in winter (January and February). This trend was already observed in *C. gallina* by Gardini and others (2000) who related it to the diminution of the clam metabolism (filtering activity) to the high temperature, sexual cycle, and eutrophication phenomena.

The mean microbial reduction during depuration was 62% for *E. coli* and 54% for FC. These data were heavily affected by the *C. gallina* samples of June and August. In these months both *E. coli* and FC increased, even if slightly, after depuration. The application of an open circuit depuration based on the use of seawater is necessarily affected by the seasonal variations of many variables, the most important of which are reported in Table 1. However, it is difficult to find a relation between the changes of these variables and the failure in the depuration of clams. In fact, both June and August

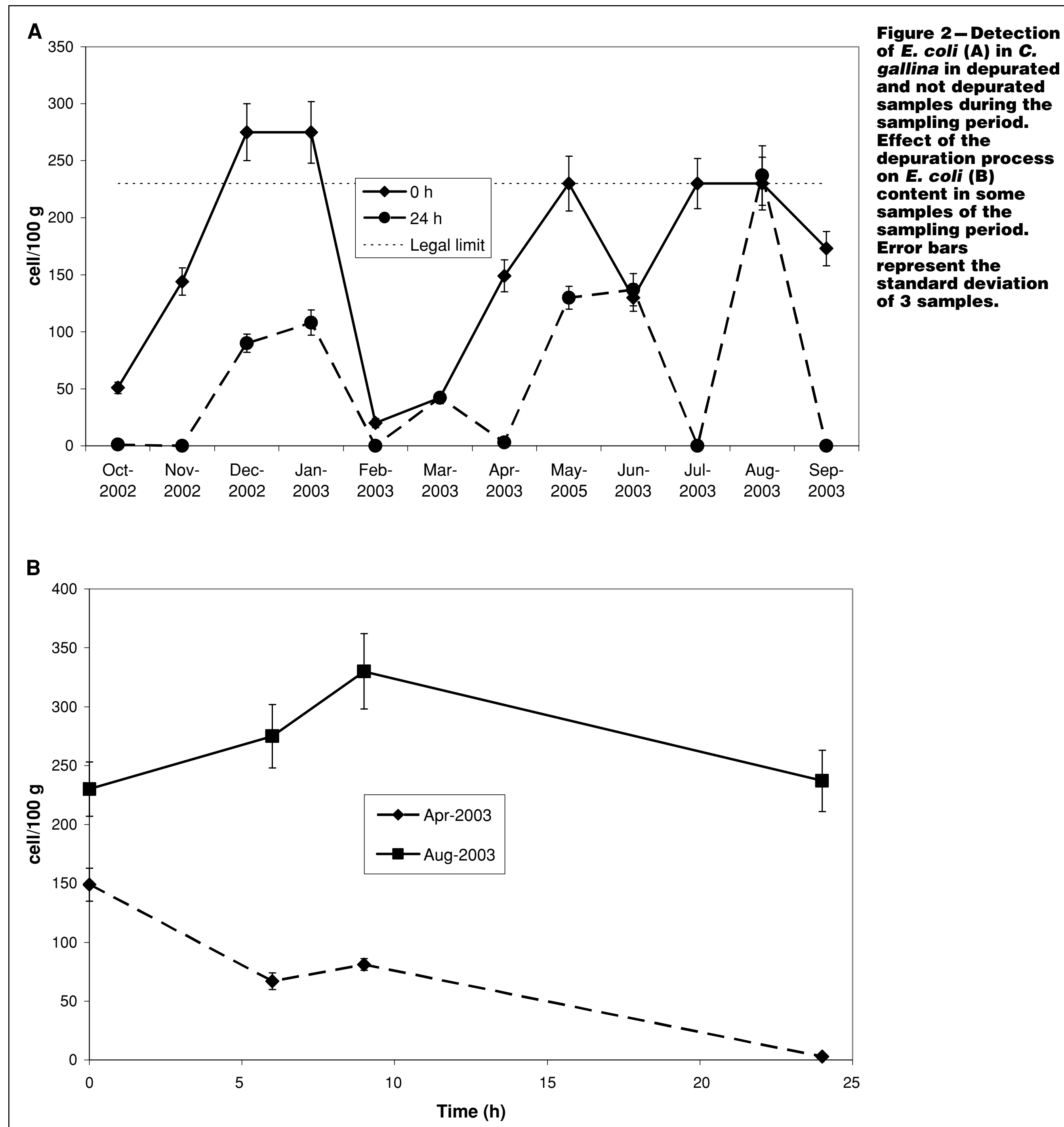

Figure 2—Detection of *E. coli* (A) in *C. gallina* in depurated and not depurated samples during the sampling period. Effect of the depuration process on *E. coli* (B) content in some samples of the sampling period. Error bars represent the standard deviation of 3 samples.

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were characterized by high temperature (about 25 °C), but an analogous temperature characterized also the depuration in July. In addition, while the seawater of June was characterized by the lower oxygen saturation (less than 50%), in August oxygen saturation was the highest (96%) observed in the period considered here. Moreover, a temperature higher than 20 °C should be favorable to a rapid removal of *E. coli* (Marino and others 1999). In general, higher temperature is favorable to the elimination of undesired bacteria but it may affect negatively the quality of the product by increasing the mortality of molluscs (Doré and others 2003). Also the salinity is reported to greatly affect the depuration efficacy (Doré and others 2003). Previous studies indicated that a salinity lower than 25% had a detrimental effect on the rate of clearance of *E. coli* in scallops, while these bacteria were successfully eliminated at values of 28% (Doré and others 2003). In the case of *C. gallina,* a clear relationship between water salinity and depuration efficacy was not found. In fact, the FC and *E. coli* counts were not univocally reduced in months in which the values of salinity were above 30%. The highest reductions both for FC and *E. coli* were observed in July samples depurated with seawater having the highest salinity value (37%). On the contrary, a salinity value of 36% was associated both to slight increases (August) and marked reductions

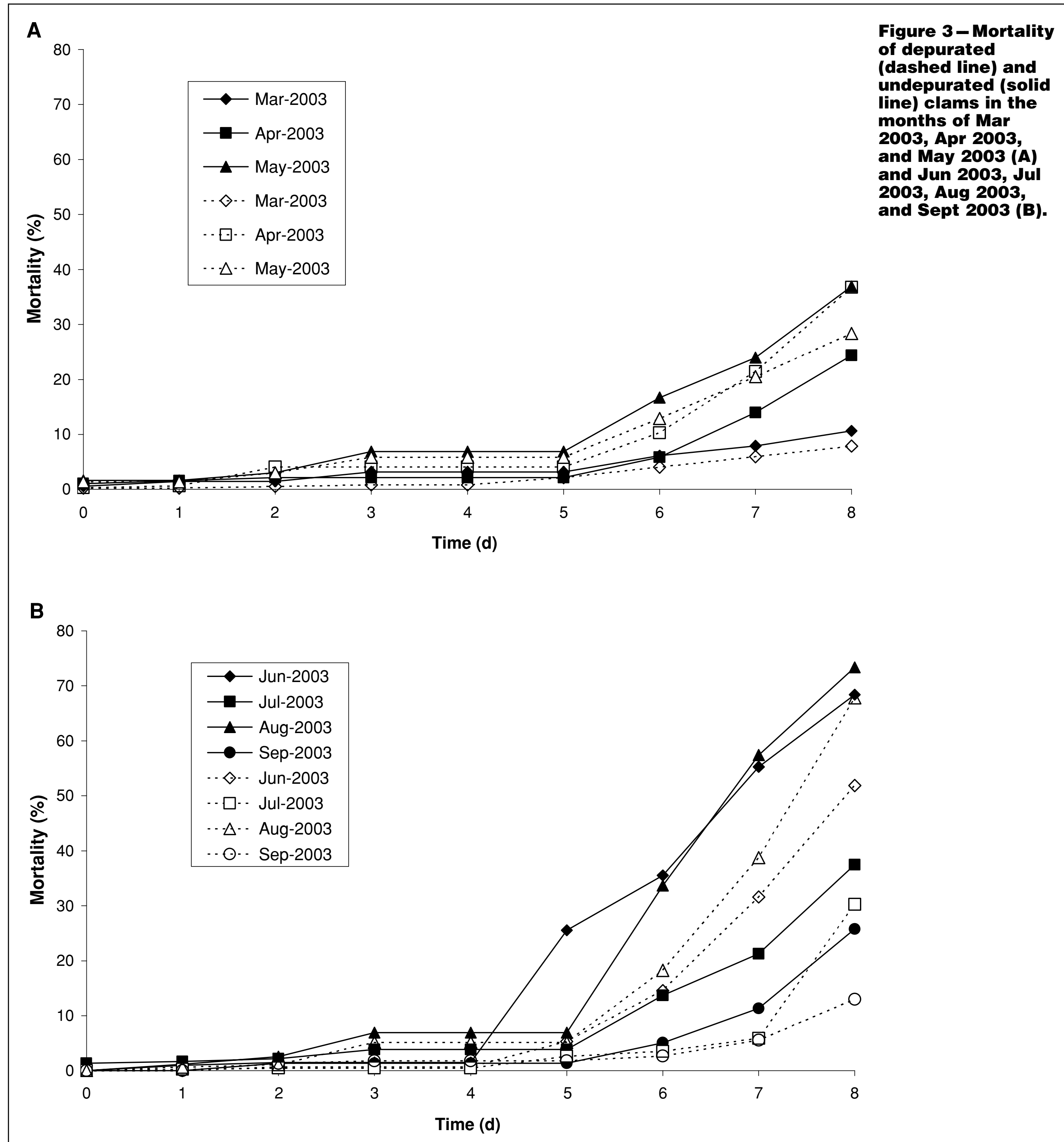

Figure 3—Mortality of depurated (dashed line) and undepurated (solid line) clams in the months of Mar 2003, Apr 2003, and May 2003 (A) and Jun 2003, Jul 2003, Aug 2003, and Sept 2003 (B).

(September and November) of *E. coli* and FC. In the clams analyzed in this study, *V. parahaemolyticus* was never detected. Nevertheless, *V. alginolyticus* was found in 2 samples of May and September. Otherwise, *V. alginolyticus* was the dominant vibrio species in mussels harvested in the Adriatic Sea (Ripabelli and others 1999) and in Turkey (Colakoglu and others 2006). The presence of this vibrio is of health concern because it can be the causative agent of gastroenteritis, even if the mechanism through which the pathogenicity is expressed is not yet clear (Oliver and Kaper 1997; Baffone and others 2001). The presence *V. alginolyticus* in the samples of September in which the depuration was particularly efficient in the reduction of FC and *E. coli* reinforced the considerations about the real significance of these 2 indicators for the evaluation of the microbiological state of seafoods, and particularly molluscs. Moreover, also in the not depurated samples the presence of this vibrio was not associated with particularly high counts of *E. coli* and FC. In fact, these counts were always lower than the legal limits imposed by EC regulation for category A seawater. On the other hand, the use of FC and *E. coli* concentrations as indicators for shellfish quality has raised several criticisms. In fact, many researchers discussed the limits of this use of FC and *E. coli* counts (Kator and Rhodes 1994; Leclerc and others 2000) because these indices of fecal contamination are not reliable indicators of the survival strategies and distribution of vibrios and also viruses in shellfishes (Ripabelli and others 1999; Cavallo and Stabili 2002; Croci and others 2002), which are considered to be the major cause of identifiable illness and death from shellfish consumption (Wittman and Flick 1995). Moreover, questions were raised also about their mean for assessing the efficacy of the depuration process. The rate at which *E. coli* is released by clams is markedly different from that of *V. cholerae* O1 and *V. parahaemolyticus*, and the decline in numbers of the former species cannot be used to indicate a decline in number of the *Vibrio* spp. (Croci and others 2002). In addition, it has been demonstrated that fecal indicator bacteria are rapidly removed by normal purification practices whereas viruses are removed less effectively (Lee and Younger 2002). In spite of this, national and international regulations are still based on these microbial indications and these limits have to be observed for marketing shellfishes.

Conclusions

The data obtained indicate that the application of the depuration conditions used for the depuration of other clams such as the Manila clam *Tapes philippinarum* can improve the quality of *C. gallina* from water of class B in terms of vitality and sand content. Moreover, a significant reduction of *E. coli* and FC was observed in specific landing periods after depuration. However, the effects of the depuration conditions adopted on microbiological quality were extremely variable, resulting both in worsening and improving of the chosen microbiological indices. The lack of a unique behavior in relation to depuration conditions underlines the need of further trials to understand the reasons for the anomalous microbiological data detected in some months and to set up a deputation protocol specific for *C. gallina* aimed to optimize its depuration time and to control the depuration cycle. Moreover, deeper investigations are necessary also to verify the effect of the depuration conditions on other microorganisms such as vibrios of major health concern in seafood.

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