

## The use of PCR applications for assessment of *Clostridium botulinum* presence in the culture environment and fish samples in the conditions of open system production from Romanian fisheries

<sup>1</sup>Tamara Mihociu, <sup>1</sup>Nastasia Belc, <sup>1</sup>Enuța Iorga, <sup>1</sup>Lavinia Rusu and <sup>2</sup>Isabelle Metaxa

<sup>1</sup>National Institute of Research & Development for Food Bioresources – IBA Bucharest, Bucharest, România; <sup>2</sup>University “Dunărea de Jos”, Galați, România.  
Corresponding author: Tamara Mihociu, tamaramihociu@yahoo.com

**Abstract.** A rapid expansion in world aquaculture has taken place in recent years due to its highest potential to answer to the consumers demands on healthy food products. The application of biosecurity in aquaculture production systems and the safety of fish and fish products, in the context of climate changes, requires investigations of the production systems and solutions for processing and preservation technologies of fish products. PCR methods will be developed using the procedure of genes fragments amplification for *Clostridium botulinum* van Ermengem, 1896 species producing toxins A, B, E and F from isolates of *C. botulinum* using extract broth TGPY (culture medium used for proteolytic strains) and TPGYT (culture medium used for non-proteolytic strains of *C. botulinum*). The results will contribute to the integral development of food safety management and the quality of fish on the fish chain focused on the Cyprinidae for humans consumption.

**Key Words:** Aquaculture, food safety, PCR.

**Rezumat.** În decursul ultimilor ani, acvacultura mondială a cunoscut o dezvoltare rapidă datorită potențialului său ridicat de a răspunde cerințelor consumatorilor pentru produse sănătoase. Asigurarea cerințelor de siguranță alimentară pentru pește și produsele pescărești necesită culegerea de informații privind cerințele de biosecuritate în sistemele de producție din acvacultură, tehnologii de prelucrare și conservare a peștelui. Vor fi dezvoltate tehnici PCR, folosind procedura amplificării fragmentelor de gene pentru tulpini toxicogene de *Clostridium botulinum* van Ermengem, 1896 tipul A, B, E și F, prin utilizarea de extracte de bulion TGPY (mediu de cultură pentru tulpini proteolitice) și TPGYT (mediu de cultură pentru tulpini non-proteolitice). Rezultatele vor contribui la dezvoltarea integrată a managementului siguranței alimentare și a calității peștelui pe lanțul piscicol focalizat pe peștele din familia Cyprinidae, destinat consumului uman.

**Cuvinte cheie:** Acvacultură, siguranță alimentară, PCR.

**Introduction.** Risk of botulism production can appears after consumption of processed or manufactured fish, through non-aggressive preservation methods, where salt or fume concentrations are reduced or water content of product is high. If, furthermore, product is packaged under vacuum, this increases risk for *Clostridium botulinum* van Ermengem, 1896 development (Doe 1998, Peck et al 2006). Fish contamination with *C. botulinum* spores can produce when increase is achieved using semi systemic technologies, when ponds have a high organic matter loading, due to nourishment in excess of fish and of the environment factors. *C. botulinum* strains can multiply in a temperature range of 3 - 32°C (Collins & East 1998). Contaminant distribution within aquatic environment is influenced by many factors and it is generated by a combination of natural and entropic variables, insufficient studied, specifics for each studied zone (Riley et al 2007). Knowledge of variables and of measurable parameter values, which can modify eco-system functionality, could be a modality for prediction of *C. botulinum* appearance within aquatic environment. Within the performed studies in the European aquatic environment it was determined presence of *C. botulinum* spores type E from 5 cfu/kg to

5 300 cfu/kg, in fish. Huss (1980) have made studies concerning contamination location with spores of *C. botulinum* in fish and they found that there are differences between spores location among fish species. Thus, species nourished with plankton are contaminated on surface, while, species nourished with zooplankton and fish, contamination is in viscera (Hyytiä-Trees 1999). Studies were performed within three Romanian piscicultural farms (Sarinasuf, Tulcea; Mălina, Galați; Cârja, Vaslui), which use semi systemic technologies, in periods with extreme temperatures, when ponds have a high organic matter loading and there are conditions for *C. botulinum* development. There are performed a number of 47 samples in hot season (August - September, 2008) and 49 samples in cold season (March, 2009). It was detected *C. botulinum* presence, strains B, E and F in sediment, water and viscera from 5 fish species of Cyprinid: *Cyprinus carpio* (Linnaeus, 1758) - common carp (with two culture forms: *C. carpio* Lausitz and *C. carpio* Aischgrund), *Hypophthalmichthys molitrix* (Cuvier et Valenciennes, 1844) - silver carp, *Hypophthalmichthys nobilis* (Richardson, 1845) - bighead carp, *Ctenopharyngodon idella* (Cuvier et Valenciennes, 1844) - grass carp. Development of PCR analysis techniques and tools facilitated elaboration of numerous protocols for molecular detection of *C. botulinum* strains, sensitive, specific and quick, in comparison with culture techniques and biological test on mice (Hyytiä-Trees 1999). PCR specificity was confirmed, for accuracy, through DNA sequencing and field analysis through electrophoresis on agarose gel (Aranda et al 1997, Marmur 1961). This study presents results of experimental verifications of two extraction methods: CTAB method (hexadecyltrimethylammonium bromide), which is a very efficient method to remove polysaccharides and polyphenols which affect DNA purity and quality and method with use of Wizard Genomic DNA Purification kit, which it can be isolated and purified DNA in two hours. Use in parallel of those two extraction methods it is not absolutely necessary, but it means a modality to select DNA with the highest purity grade. Separation of PCR amplicons is made by electrophoresis in agarose (1.5 %, TBE 0.5x, 5v/cm), which contains ethidium bromide 0.5 µg/ml; using 10 µl reaction product and 2 µl loading buffer. Molecular mass of resulted amplicons is compared with DNA of 100 pb. Amplicons length for each gene type is: Type B 492 pb, Type F 1137 pb, Type E 410 pb (Haim & Timothy 1998, Sambrook et al 1989).

In order to establish selection criteria of the analyzed zones and of presence evaluation of *C. botulinum* strains it was considered conceptual model of Hedrick (1998), concerning causality between eco-system status and its response to events:

- evaluation of variable environmental parameters which can produce modifications in space and time of eco-systems, and can be favourable to growth and development of *C. botulinum*.
- how temperature modification affects nutrition on trophy chain and infection conditions of fishery products.

As we said before there were performed 47 samples in hot season (August - September, 2008) and 49 samples in cold season (March, 2009). Quality parameters of aquatic environment of fresh water are given by mode for measuring of chemical, biological and microbiological parameters, and modality of their changes as well during day as well during entire year. Concentration values of these parameters can describe pollution degree and biotic status of water, which can give indices and can predict probability of presence of some micro organisms. An important aspect of water quality evaluation is sampling. In case of aquaculture in open system natural ponds can be very large and can support a complex eco-system, in which the environmental parameters are very different in all three physical dimensions and in time.

**Materials.** Research was performed on major components of aquatic environment.

**Selection of piscicultural farms** was made based on the following criteria:

- bio-geographical areas from hydrographical water basins Siret, Prut and Danube, with important production of cyprinid in open system aquaculture;
- geological inputs: river beds, small variety of geological layers;
- anthropic inputs: industrial, agricultural activities, adjoining settlements;

- relationship concerning incidence of *C. botulinum* and geographical position, pollution degree given by human activities on Prut medium course, Siret inferior course and wet zone Danube Delta.

Three piscicultural farms were selected for study, according to selection criteria:

- F.1 Piscicultural farm Sarinasuf, Tulcea County, in Danube Delta, with input from Danube, through Lipoveni channel. Total area of farm S = 540 ha. Assurance of natural nourishment, plankton development is made with mineral fertilizers (ammonium nitrate). Position GPS: N = 44° 99' 78'' E = 029° 08' 50'';

- F.2 Piscicultural farm Mălina, Galați County, with input from Siret River, Danube affluent. Total area of farm S = 127 ha, sampling was made into a basin with S = 30 ha. Basin soil is sandy-argillaceous (30 % sand and 60 % clay, with rich content of humus). Assurance of natural nourishment, plankton development is made with mineral fertilizers (ammonium nitrate). Position GPS: N = 45° 40' 705 E = 027° 95' 367'';

- F.3 Piscicultural farm Cârja, Vaslui County, with input from Prut River, Danube affluent. Total area of farm S = 647 ha, sampling was made into a basin with S = 297 ha. Assurance of natural nourishment is made through initiate of industrial culture of infusorians. Feeding is made daily, on periods with temperatures higher than 10°C, with feeds prepared into farm, using raw materials: meal of sun flower, wheat, barley, maize, fodder calcium. Position GPS: N = 46° 15' 849'' E = 021° 11' 231''.

#### **Selection criteria of environment variables:**

- change of seasonal models, which through precipitations and water leak produce effects on hydrological characteristics of aquatic systems, population of sub-aquatic organisms being sensitive to changes concerning frequency, time and events calendar within periods with extreme values;

- increase/decrease of water masses temperature - metabolic rhythm of some organisms, as well as global productivity of aquatic eco-systems is directly regulated by temperature. Increase of water temperature and reduction of water masses flow (into cold season) have as effect increase of nutrients level in water, reducing water quality with consequences on increase of bacteriological risk.

- atmospheric inputs which affect biological parameters of aquatic environment quality.

**Selection criteria of environmental variable parameters**, measurable, were the following:

- change of seasonal models - sampling was made in hot season (25.08 - 04.09.2008), between 11 a.m. 14 p.m., with starting of alga bloom and in cold season (04.03 - 10.03.2009), at snow melting, within periods without solar exposure, with precipitations;

- increase/decrease of water masses temperature, in hot season water temperature was in the range 21°C–27.4°C (from N=46°15' to N=44°99'), periods without precipitations, and in the cold season temperature of aquatic environment was in the range 6°C–7.8°C (from N=46° 15' to N=44° 99'), periods with precipitations and large flows of storm water;

- atmospheric inputs which affect biological parameters of aquatic environment quality:

- oxygen is the most important constitutive element in chemistry of surface waters. Oxygen solubility in water is inversely proportional with temperature and directly proportional with grade of aquatic vegetation,

- luminosity degree related to water turbidity, regardless of its nature and close to temperature, determines a well-balanced sheet between semi-obscure phenomena, those which arise in the dusk and the photosynthetic ones,

- water pH at surface and basin deep.

**Parameters of aquatic environment** from ponds were measured at water surface and at surface of oozy layer (Table 1 in hot season and Table 2 in cold season), Measurements were performed with laboratory oxygen meter IntelliCAL (measurement range: 0.1–20.0 mg/l, 1-200 % saturation; pHC 301: 0.0–14.0 pH; temperature 0–50°C).

Table 1

Parameters measured into aquatic environment in sampling points in hot season

Farm	Measured parameters			
	Measurement level (m)	Temperature (°C)	Dissolved oxygen (mg/l O <sub>2</sub> )	pH
Sarinasuf, Tulcea	0.25 – 1.35	25.8 – 27.4	0.2 – 13.66	7.29 – 8.23
Mălina, Galați	0.25 – 2.20	24.7 – 26.4	0.15 – 7.95	7.15 – 7.83
Cârja, Vaslui	0.25 – 1.2	19.3 – 22.5	4.45 – 13.15	7.29 – 8.43

Table 2

Parameters measured into aquatic environment, in sampling points in cold season

Farm	Measured parameters			
	Measurement level (m)	Temperature (°C)	Dissolved oxygen (mg/l O <sub>2</sub> )	pH
Sarinasuf, Tulcea	0.50 – 1.30	6	-	-
Mălina, Galați	0.25 – 1.80	7.5 – 8.2	10.40 – 12.64	7.83 – 8.21
Cârja, Vaslui	0.25 – 1.3	6.5 – 6.9	9.07 – 11.90	7.82 – 8.19

In order to determine presence of *C. botulinum* sampling plan included: in hot season a sample of surface water, two samples of sediment, a sample of water suspensions caught on gauze cartridge, through filtration of 50 l water. There were sampled, from those three farms, 24 sediment samples, 12 water samples, 12 sediment water samples in different sampling points: input, center, bank sides and ejection (Figure 1 - Sarinasuf farm; Figure 2 - Mălina farm; Figure 3 - Cârja farm).

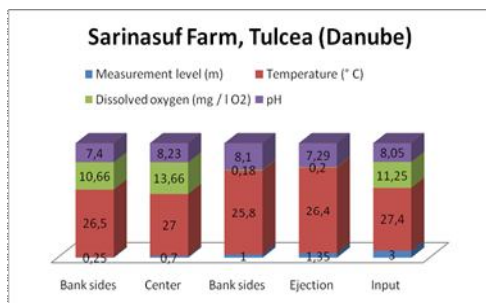


Figure 1. Parameters measured into aquatic environment in Sarinasuf Farm in hot season

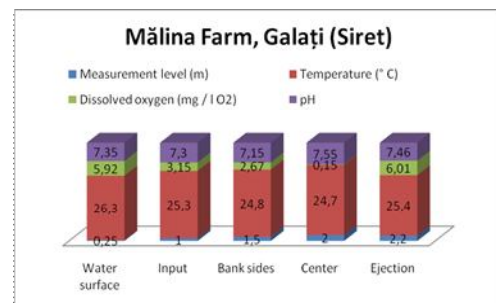


Figure 2. Parameters measured into aquatic environment in Mălina Farm in hot season

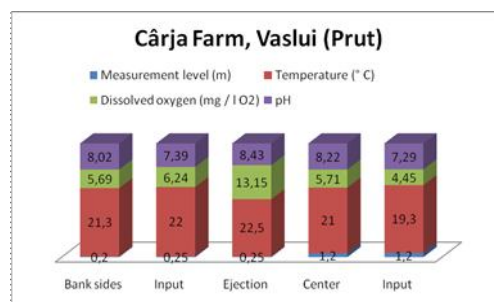


Figure 3. Parameters measured into aquatic environment in Cârja Farm in hot season

In cold season it was taken into consideration a possible concentration of bacterial activity into sediment of pond, sampling plan being modified through increase of number of sediment samples to 4 in a sampling point and chosen of sampling points taking into consideration the following: height of water layer, streams, submerge vegetation, vicinity of bank sides (Figure 4 - Mălina farm; Figure 5 - Cârja farm).

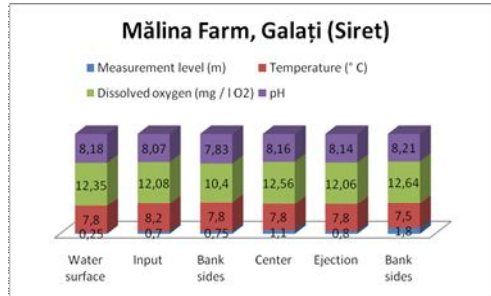


Figure 4. Parameters measured into aquatic environment in Mălina Farmin cold season

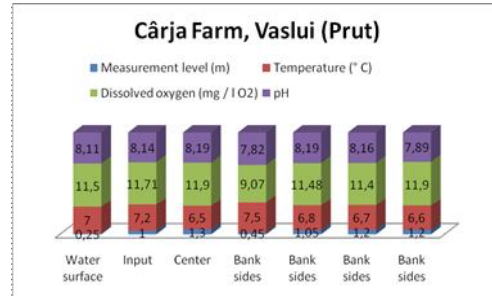


Figure 5. Parameters measured into aquatic environment in Cârja Farm in cold season

Thus, samples were the following:

- Sarinasuf, Tulcea - 16 samples;
- Mălina, Galați - 14 samples;
- Cârja, Vaslui - 19 samples.

Water was sampled according to SR ISO 5667-4:2000 and water suspensions were sampled according to SR ISO 5667-6:1997, sediment was extracted with laboratory dredge according to SR ISO 6107-2:1997 and SR ISO 5667-12:2001, fish sampling according to SR EN 14962:2006.

Samples were prelevated once from each farm, in each season.

Among fish species produced into farms there were made determinations from viscera of species: *C. carpio* Lausitz, *C. carpio* Aischgrund, *H. molitrix*, *H. nobilis*, *C. idella*. Sample consisted into viscera of five fish of same species and same age.

Samples in hot season were constituted from:

- Cârja Farm, Vaslui, three samples from *C. carpio* Lausitz, *H. molitrix* and *C. idella* (fish with weight of 1.5 – 2.5 kg);
- Mălina Farm, Galați, three samples from *C. carpio* Lausitz, *H. molitrix* and *H. nobilis* (fish with weight of 0.8 – 1.3 kg);
- Sarinasuf Farm, Tulcea, three samples from *C. carpio* Lausitz, *C. carpio* Aischgrund and *C. idella* (fish with weight of 1.5 – 2.5 kg).

Samples in cold season were constituted from:

- Cârja Farm, Vaslui, three samples from *C. carpio* Lausitz, *H. molitrix* and *C. idella* (fish with weight of 1.5 – 2.5 kg);
- Sarinasuf Farm, Tulcea, two samples from *C. carpio* Lausitz and *C. idella* (fish with weight of 1.5 – 2.5 kg).

## Methods

### Determination of *C. botulinum* through PCR techniques

Strains of *C. botulinum* from group I are proteolytic and produce toxins A, B and F, or a mixture of them. There have an optimal growing at 35-40°C and produce spores very resistant to heat action ( $D_{112}^*$  time of 1.23 minutes). There are developed on animal protein, producing ammonia and hydrogen sulphide, also fermenting some saccharides. Increase is inhibited by NaCl concentrations of 6.5 % and pH of 8.5.

Strains of *C. botulinum* from group II are non-proteolytic, saccharolytic and produce toxins B, E or F. These are psychrotrophic bacteria with optimal temperature for growing of 18-25°C. Their spores are a little thermo-resistant and  $D_{80}$  is in the range 0.6

and 1.25 min. In fact, spores resistance at heat is underestimating, moderate temperatures being sufficient for destruction of enzyme necessary for germination. Spores rest viable, but there are unable to germinate. Increase is stimulated by fermentescible saccharides, being inhibited by 6.5 % NaCl and pH 8.5.

**Culture medium used:**

- Trypticase-peptone-glucose-yeast extract broth (TPGY), culture medium used for proteolytic strains of *C. botulinum*;

- Trypticase-peptone-glucose-yeast extract with trypsin broth (1:250) (TPGYT), culture medium used for non-proteolytic strains of *C. botulinum*.

**DNA extraction.** There were inoculated 1 ml liquid sample and 1 g solid sample, respectively in 10 ml medium TPGY/ TPGYT in anaerobe conditions (through slopping of paraffin stopper) at 28°C (TPGY), 35°C (TPGYT), respectively, time of 24 hours. 1.4 ml of culture medium from each sample were centrifuged (14,000 xg, 2 minutes), and sediment was extracted. Bacterial cells were washed with 1 ml PBS, pH 7.4 and centrifuged at 14,000 xg for 2 minutes. Sediment was then washed with a solution of 400 µl PBS, pH 7.4 and 100 µl lysozyme (10 mg lysozyme/ml TE (10 mM Tris, 1 mM EDTA) pH=7.4). Tubes were then incubated on a water bath with stirring for 15 minutes at 37°C. Then there were added 10 µl proteinase K (10 mg Pk/ml TE), and tubes were incubated on water bath 60 minutes at 60°C and 10 min at 100°C. After incubation tubes were centrifuged at 14,000 xg for 2 minutes. 1.5 ml supernatant were treated with 50 µl natrium acetate 3 M and 1 ml ethanol 95 %, tube being then incubated 30 min at -70°C. Then tube was centrifuged at 14,000 xg for DNA sedimentation. Sediment was dried, rehydrated with 50-100 µl ultra pure water and stored at -20°C.

**Primers.** Used primers were those produced by Haim & Lilly (1998). Sequences of advancement primers (F) and of reversion (R) are:

- Type B:

F 5' -GAG ATG TTT GTG AAT ATT ATG ATC CAG -3'

R 5' -GTT CAT GCA TTA ATA TCA AGG CTG -3'

- Type E:

F 5' -CCA GGC GGT TGT CAA GAA TTT TAT -3'

R 5' - TCA AAT AAA TCA GGC TCT GCT CCC -3'

- Type F:

F 5' -GCT TCA TTA AAG AAC GGA AGC AGT GCT -3'

R 5' -GTG GCG CCT TTG TAC CTT TTC TAG G-3'

**PCR reaction.** Each set of primers was used in separated reactions. Reaction was performed within 25 µl reaction volume which contain 1X PCR buffer, 2.5 mM MgCl<sub>2</sub>, 0.5 µM each primer, 0.025 U/µl DNA polymerase and 1 µl DNA. Amplification conditions were the following: initial denaturation 95°C for 5 min, denaturation at 94°C-1 minute, alignment 60°C-1 minute and extension 72°C-1 minute (all for 30 cycles), followed by final extension at 72°C for 10 minutes.

**Visualisation in agarose gel.** Separation of PCR amplicons was achieved through electrophoresis in agarose (1.5 %, TBE (Trisodic acid-Na<sub>2</sub>EDTA) 0.5X, 5v/cm), which contains ethidium bromide 0.5 µg/ml, through using of 10 µl reaction product and 2 µl loading buffer. Molecular mass of the resulted amplicons was compared with DNA of 100 pb. Amplicons length for each type of gene is: Type F 1137 pb, Type B 492 pb, Type E 410 pb.

**Results.** Determinations result in hot season (August - September, 2008) shown presence of *C. botulinum* type E, non-proteolytic, in water; water suspension and sediment (Sarinasuf farm, Tulcea) and in sediment (Mălina farm, Galaţi). Measured aquatic environmental parameters were those in Table 3.

Table 3

Results of *C. botulinum* strains determination in hot season

Farm	Sample	Type F	Type B	Type E	Aquatic environmental parameters at sampling
Sarinasuf, Tulcea	Water	-	-	TGPYT	0.25 m
	Water suspension	-	-	TGPYT	26.5 – 27.4 <sup>0</sup> C 10.66 mg/l O <sub>2</sub> pH = 7.4
	Sediment	-	-	TGPYT	1 – 1.35 m 25.8 – 26.4 <sup>0</sup> C 0.18 – 0.2 mg/l O <sub>2</sub> pH = 8.1 – 7.29
Mălina, Galați	Water	-	-	-	2 m
	Water suspension	-	-	-	24.7 <sup>0</sup> C 0.15 mg/l O <sub>2</sub>
	Sediment	-	-	TGPYT	pH = 7.55

Determinations resulted in cold season (March, 2009) shown presence of *C. botulinum* type E, non-proteolytic, in sediment and in viscera of *H. molitrix* (Cârja farm, Vaslui) and in sediment (Sarinasul farm, Tulcea). Measured aquatic environmental parameters were those in Table 4.

Table 4

Results of *C. botulinum* strains determination in cold season

Farm	Sample	Type F	Type B	Type E	Fish species	Aquatic environmental parameters at sampling
Cârja, Vaslui	Sediment	-	-	TGPYT	-	0.45 m 7.5 <sup>0</sup> C 9.07 mg/l O <sub>2</sub> pH = 7.82
	Fish viscera	-	-	TGPYT	<i>H. molitrix</i>	
Sarinasuf, Tulcea	Sediment	-	-	TGPYT	-	0.6 m 6 <sup>0</sup> C

Reaction products obtained in PCR Eppendorf equipments were transferred into agarose gel for results evidence. In order to establish size of amplicons of PCR reaction it was used a marker with molecular mass of 100pb. Amplicons of 410pb present in *C. botulinum* type E in: Figure 6 - viscera of *H. molitrix* species, Cârja farm, Vaslui; Figure 7 - Sarinasuf farm pond sediment, Tulcea; Figure 8 - Cârja farm pond sediment, Vaslui (samples in cold season).

**Discussion.** It was taken into consideration that the aquatic environmental parameters to be favourable increase of *C. botulinum*, in hot periods during summer and periods without sun exposure in winter.

In hot season it was determined presence of *C. botulinum* type E of group II in sediment, water and water suspension. Sampling was performed in hot periods. Aquatic environment was characterized through:

- high water temperatures 19.4–27.4<sup>0</sup>C;
- dissolved oxygen = 0.15–13.66 mg/l O<sub>2</sub>;
- pH with values in the range 7.15–8.43;
- high organic matter content: algae;

- ponds with adjoining settlements and industrial activity.

In the cold season was determined presence of *C. botulinum* type E of group II in sediment and viscera of fish species *H. molitrix*. Sampling was made in periods without sun exposure. Aquatic environment was characterized through:

- water temperatures of 6–8.2°C;
- dissolved oxygen = 9.07–12.64 mg/l O<sub>2</sub>;
- pH = 7.82–8.21;
- high organic matter content: transparent = 18 cm;
- ponds with adjoining settlements.

Evaluation of *C. botulinum* strains presence, in Romanian piscicultural farms, can contribute to elaboration of Good Manufacturing Practices, of HACCP Plan, as well in primary production as well on food chain of fish.

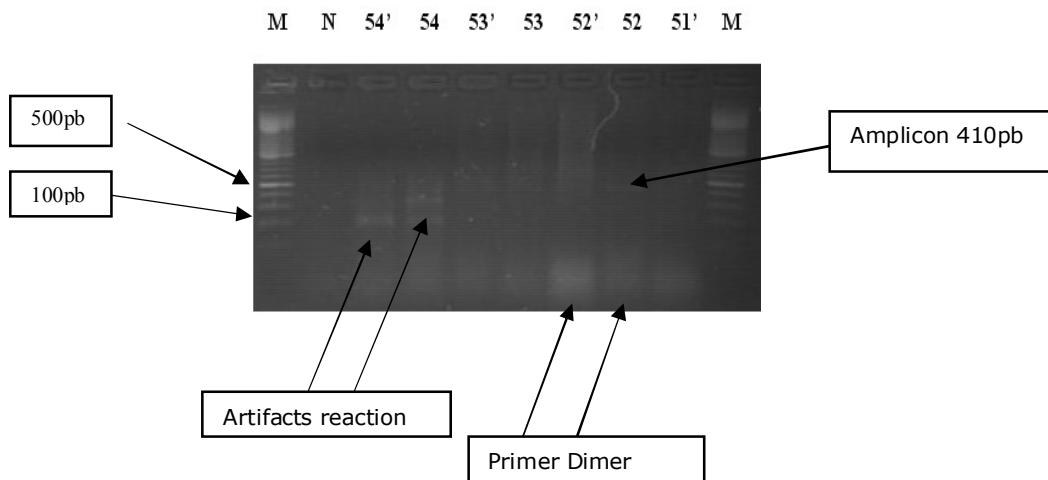


Figure 6. An agarose gel image where the amplicons were revealed by *C. botulinum* E strain (51-54 - samples, N - control, M - marker 100pb) (culture medium TPGYT).  
Viscera *H. molitrix*, Cârja farm, Vaslui

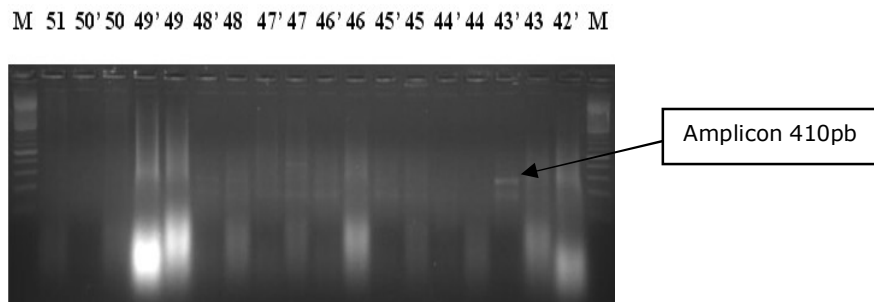


Figure 7. An agarose gel image where the amplicons were revealed by *C. botulinum* E strain (42-51 - samples, N - control, M - marker 100pb) (culture medium TPGYT)  
Sarinasuf farm pond sediment, Tulcea



M 16' 16 15' 15 14' 14 13' 13 12' 12 11' 11 10' 10 9' 9 8' 8 M

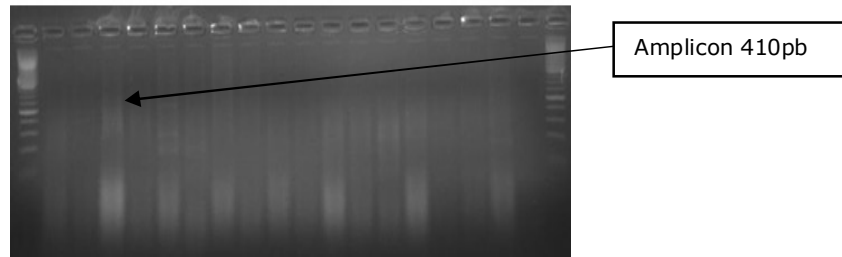


Figure 8. An agarose gel image where the amplicons were revealed by *C. botulinum* E strain (8-16 - samples, N - control, M - marker 100pb) (culture medium TPGYT) Cârja farm pond sediment, Vaslui

**Conclusions.** *C. botulinum* represents a hazard for food safety of food stuffs processed through modern technologies. Fishery products minimal processed, with a minimum adding of preservatives, packaged under vacuum or modified atmosphere, stored at refrigeration temperatures in order to assure a long preservation period, can increase the risk of pathogenic anaerobic bacteria development, *C. botulinum* type.

Aquaculture in open system is associated with environmental problems, which present hazards and risks concerning fishery products safety, being vulnerable to climate changes.

The most significant hazards are those biological and chemical ones. Risk analysis of these hazards brings benefits for sustainability, rent ability and efficiency of fish sector.

Qualitative assessment performed within this study, concerning exposure of fishery products to contamination with *C. botulinum* of groups I and II, comes to complete the available data about this pathogen in this economic sector.

Quantitative assessment of the exposure it was focused on identification of pathogen agent in point where it come in food stuff, growing basins of three Romanian piscicultural farms:

- Sarinasuf, Tulcea Position GPS: N = 44° 99' 78'' E = 029° 08' 50'';
- Mălina, Galați Position GPS: N = 45° 40' 705'' E = 027° 95' 367'';
- Cârja, Vaslui Position GPS: N = 46° 15' 849'' E = 021° 11' 231''.

There were assessed the major components of the aquatic environment: water, water suspension, sediment and fish. There were used PCR techniques for detection of *C. botulinum*, strains of groups I and II.

The results of this study revealed presence of *C. botulinum* type E, non-proteolytic, in all components of aquatic environment of those three farms.

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Authors:

Tamara Mihociu, National Institute of Research&Development for Food Bioresources - IBA, Interdisciplinary Researches Department, Bucharest, Romania, Bucharest 2, 6 Dinu Vintilă Street, CP 021102, e-mail: tamaramihociu@yahoo.com

Nastasia Belc, National Institute of Research&Development for Food Bioresources - IBA, Interdisciplinary Researches Department, Bucharest, Romania, Bucharest 2, 6 Dinu Vintilă Street, CP 021102, e-mail: nastasia.belc@bioresurse.ro

Enuța Iorga, National Institute of Research&Development for Food Bioresources - IBA, Interdisciplinary Researches Department, Bucharest, Romania, Bucharest 2, 6 Dinu Vintilă Street, CP 021102, e-mail: enutai@yahoo.com

Lavinia Rusu, National Institute of Research&Development for Food Bioresources - IBA, Interdisciplinary Researches Department, Bucharest, Romania, Bucharest 2, 6 Dinu Vintilă Street, CP 021102, e-mail: lavinia.rusu@bioresurse.ro

Isabelle Metaxa, University "Dunărea de Jos" Galați, Department of Aquaculture, Environmental Science and Cadastrem, Romania, Galați, 47 Domneasca Street, CP 800008, e-mail: Isabelle.Metaxa@ugal.ro

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