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Okhotsk Sea ice as a reservoir of *Listeria monocytogenes*

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Abstract: The contamination of sea ice by *Listeria monocytogenes* was investigated in the western part of the Sea of Okhotsk for the first time. The influence of the Amur River outflow on *Listeria*'s distribution on the northeastern Sakhalin shelf and slope is discussed. Laboratory investigations of the survival of *L. monocytogenes* in frozen seawater are provided as well. It is possible to characterize the temporal evolution of the reproductive function of *L. monocytogenes* in sea ice. The duration of *Listeria*'s reproductive period in sea ice depends on the strain's biological peculiarities and on the temperature regime. Colder temperatures result in a long-term reproductive ability of *L. monocytogenes* in sea ice.

key words: Listeria monocytogenes, sea ice, Sea of Okhotsk, distribution, reproductive ability

Introduction

At present, sea ice is frequently regarded as an important monitoring component for anthropogenic pollution of the hydrosphere. There have been numerous investigations into the role of sea ice in the accumulation and migration of pollutants (Pfirman *et al*., 1995; Rigor and Colony, 1997; Ivanov, 1998; Kondratieva, 2002; Korsnes *et al*., 2002). However, the question of how pathogenic bacteria survive in sea ice has not received adequate attention. In our view, knowledge of the agents of saprozoonoses is of special importance. These microorganisms are capable of surviving and propagating not only in a hematothermal organism, but also in the environment. The bacteria *Listeria monocytogenes* belongs to this group.

L. monocytogenes is the etiological agent of listeriosis, a serious infection in humans and animals. During the 1980s, listeriosis became one of the five leading and most

dangerous alimentary bacterial infections (Liston, 1990). The increasing number of listeriosis outbreaks, caused by the consumption of infected seafood (Brett *et al*., 1998; Rocourt *et al*., 2000; Gram, 2001), dictates the necessity of a more detailed investigation of *L. monocytogenes* marine ecology. The importance of microbiological research on *L. monocytogenes* in the Sea of Okhotsk is obvious, as the proportion of products from the Okhotsk in the Asian food ration grows from year to year.

In the following sections the occurrence and distribution of *L. monocytogenes* bacteria in sea ice of the western part of the Sea of Okhotsk and experimental investigations of *Listeria*'s reproductive function in frozen seawater are presented and discussed.

Materials and methods

This study is a microbiological part of the joint German-Russian projects KOMEX I and II (Kurile Okhotsk Sea Marine EXperiment). The material was collected on an ice expedition (ICE1) during the ice-covered season in March 1999.

The area of investigation covers the northeastern Sakhalin shelf and slope. This region is situated near one of the largest rivers in East Asia, the Amur River, which plays an important role in the freshwater balance of the Sea of Okhotsk (Fig. 1a). Moreover, a huge amount of suspended organic matter and minerals is transported by the Amur freshwater outflow to the western part of the Sea of Okhotsk.

During winter the ice cover of this area is not permanent, but consists of different sized ice floes which are gradually replaced by open water. We used an MI-8 helicopter for collecting the samples under these ice conditions. It allowed us to move from one ice field to another safely. All sampling was done from drifting ice. During the observation period the thickness of the ice cover did not exceed 0.8–1 m. Ice samples were collected using a hand driller (core diameter: 0.18 m). Ice cores were sampled every 0.15 m. The melting of ice samples took place at temperatures of 8–10°C. Prevention of man-made *Listeria* contamination was ensured by the sterile state of sea ice sampling procedures.

An original enrichment method for *L. monocytogenes* accumulation in samples was applied (Terekhova *et al*., 2004). *L. monocytogenes* cultures were isolated according to the methodical directions "MIN 4.2.1122-02" (2002) using *Listeria*'s selective medium (Bakulov and Vasilyev, 1999). The culture was identified according to the criteria of Bergey's Manual of determinative bacteriology (1997).

For the laboratory experiments the following *L. monocytogenes* reference-strains (serotype 1/2a) were used: **10CN** (isolated from silo), **P** (isolated from soil), **2M** (isolated from a human patient) and **1106** (isolated from seawater). These strains were obtained from the All-Russian State Research Institute for Control Standardization and Certification of Veterinary Preparations (Moscow), except for strain **1106** which was isolated by us from Okhotsk Sea water and which has typical cultural-morphological, biochemical and antigenic properties.

To examine the duration of the bacterial reproductive period in sea ice, filtered and unfiltered seawater were inoculated by *L. monocytogenes*. The infecting dose was 1000 cells in 1 m*l*. The inoculated water was separated (3 m*l* per vial), slowly frozen and

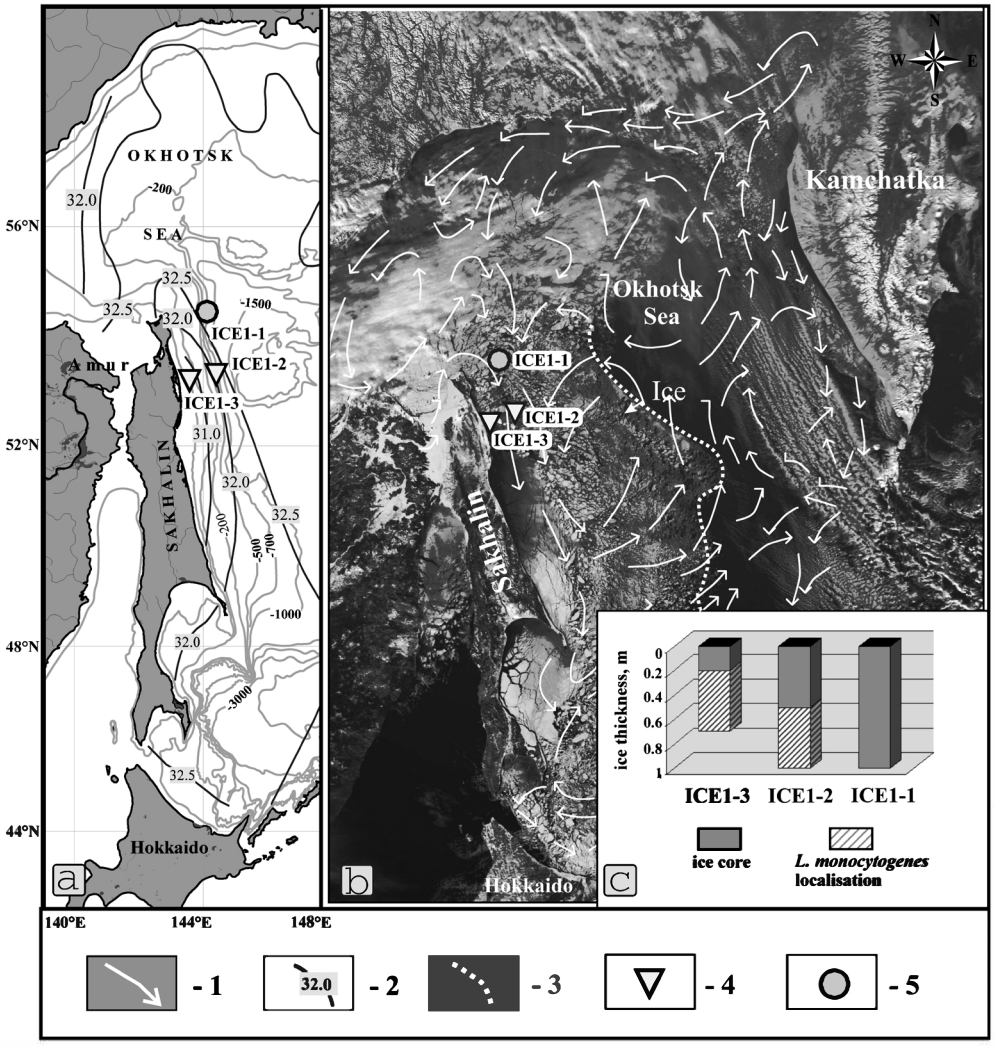

Fig. 1. (a) Listeria findings in the northwest part of the Sea of Okhotsk; (b) Okhotsk Sea ice cover at the end of the 1999 winter season (satellite image NOAA: http://snow.civil.kitami-it.ac.jp/satellite.htm); (c) Listeria distribution in the ice cores. Legend: 1—currents; 2—salinity, psu; 3—ice field border; ICE1 stations; 4—*L. monocytogenes* detected; 5—*L. monocytogenes* not detected.

exposed for six months to two thermal regimes: −4 to −6°C and −20 to −22°C. These temperature ranges were chosen based on the average temperature of the upper and lower sea ice boundaries in the northwestern part of the Sea of Okhotsk (Doronin, 1978). The six-month period of sample exposure corresponds to the average ice-covered winter period in the western part of the sea. On fixed dates (1, 2, 3, 7, 14, 30, 60, 100, 120 days) the ice samples were slowly (over 24 hours) thawed. The obtained substrates (0.1 m*l*) were seeded into Petri dishes with 30 m*l* *Listeria*'s selective medium.

Inoculated Petri dishes were incubated at 37°C for one or two days. Bacterial amount is expressed as the decimal logarithm of the number of *Listeria* clumps per unit volume of thawed water (Log CFU/m*l*).

Results and discussion

Field research

Our investigation was based on 17 ice samples from three oceanographic stations. A total of six strains of *Listeria monocytogenes* were isolated from these samples, which were identical in their morphological, cultural, biochemical and antigenic properties. Thus, the contamination of the sea ice offshore of northeastern Sakhalin by *L. monocytogenes* was verified.

The distribution of *L. monocytogenes* in the studied ice cores was not homogeneous (Fig. 1b). *Listeria* was detected throughout the entire ice core (apart from its thin upper layer) and in the water column beneath the ice over the shelf near the NE Sakhalin coast (ICE1–3, depth 50 m). At the slope station (ICE1–2, depth 540 m) bacteria were found only in the lower part of the ice core. And at the northernmost offshore station (ICE1–1, depth 734 m) bacteria were not observed either in the ice core or in the water column.

Thus, the features of *Listeria*'s distribution in the ice cores allow us to establish discrete distribution patterns in the surface waters of the western Sea of Okhotsk. During winter, new sea ice increases its thickness predominantly by growth on its lower boundary. Sea ice is closely related to seawater and its microflora is significantly determined by the seawater's microflora. Such circumstances, together with well known features of water circulation and the general pattern of ice drift, permit us to draw some conclusions about the area where the ice forms (Fig. 1c).

The absence of *Listeria* in the upper part of drifting ice floes at stations ICE1–2 and ICE1–3 indicates that those ice floes originate in the northern, *Listeria*-free region of the Sea of Okhotsk. The contamination of the ice occurs later, when floes, drifting southward, reach waters infected by bacteria. The uniform distribution of *L. monocytogenes* in the lower part of the ice cores (ICE1–2, ICE1–3) and its detection in the surface waters (ICE1–3) demonstrates that tested ice floes had not left the contaminated area before sampling.

Drifting sea ice in the northern and northwestern parts of the Sea of Okhotsk, after its formation, moves southward due to the dominant winds and general cyclonic water circulation (Rostov *et al*., 2001). Near the northern edge of Sakhalin Island drifting ice floes are influenced by the Amur River outflow.

Thus, it is possible to conclude that contamination of sea ice by *L. monocytogenes* is due to the influence of the polluted Amur River outflow. In contrast, the absence of bacteria in station ICE1–1, farthest offshore, shows that this area is not affected by the polluted Amur outflow. Unfortunately, there are no historical data on Amur River direct pollution by *L. monocytogenes.* This remains a main objective for further field investigations.

Laboratory research

To investigate the survival of *L. monocytogenes* in sea ice, the dynamics of *Listeria*'s reproductive forms in frozen unfiltered seawater were examined. Laboratory modeling demonstrated significant differences in the duration of the reproductive period of *L. monocytogenes* strains isolated from different objects (Table 1).

Strain **2M**, isolated from a human patient, kept its reproductive ability in frozen seawater up to 30 days. The reproductive period in frozen seawater of strain **P**, isolated from soil, and strain **10CN**, isolated from silo, continued up to 60 and 90 days, respectively. Strain **1106**, isolated from Okhotsk seawater, conserved reproductive properties under the same conditions during the entire exposure (6 months).

Temperature also has a significant impact on the reproductive ability of *L. monocytogenes* in sea ice. All examined strains of *Listeria* under the influence of low negative temperatures (-20 to -22°C) had a higher reproductive potential than in the range -4 to -6°C. It is likely that colder temperatures considerably depress the vital activity processes of concomitant sea ice microflora, and reinforce the competitive ability of *Listeria*, which has psychrophilic features.

This prediction was verified experimentally. The dynamics of several of reproductive forms of the same *Listeria* strains in filtered frozen seawater with the analogous

Table 1. Dynamics of *Listeria monocytogenes* reproductive forms in frozen **unfiltered** marine water at -20~-22°C.

Exposure time (days)	Log CFU/m*l* ± SE							
	Strain **2M**		Strain **10CN**		Strain **P**		Strain **1106**	
	−20~−22°C	−4~−6°C	−20~−22°C	−4~−6°C	−20~−22°C	−4~−6°C	−20~−22°C	−4~−6°C
1	3.84 ±0.21	3.15 ±0.20	3.35 ±0.21	3.49 ±0.21	3.59 ±0.21	3.54 ±0.17	3.55 ±0.18	3.58 ±0.21
2	3.45 ±0.19	3.12 ±0.19	3.25 ±0.18	3.18 ±0.12	3.49 ±0.19	3.32 ±0.20	2.42 ±0.19	3.64 ±0.22
3	3.38 ±0.19	3.04 ±0.16	3.11 ±0.19	2.45 ±0.19	3.32 ±0.07	3.10 ±0.14	3.20 ±0.20	2.71 ±0.12
7	3.37 ±0.14	2.86 ±0.11	3.80 ±0.15	2.28 ±0.07	3.03 ±0.16	2.73 ±0.12	2.30 ±0.05	2.65 ±0.06
14	2.18 ±0.07	1.42 ±0.06	2.41 ±0.06	0.89 ±0.09	2.64 ±0.05	2.71 ±0.07	2.30 ±0.04	2.22 ±0.02
30	0.65 ±0.01	0.41 ±0.01	2.19 ±0.02	0.85 ±0.05	2.75 ±0.13	2.31 ±0.05	2.45 ±0.06	1.52 ±0.02
60	0	0	2.11 ±0.01	0.65 ±0.06	0.32 ±0.01	0.1 ±0.01	2.45 ±0.03	1.50 ±0.02
90	0	0	1.00 ±0.01	0.12 ±0.01	0	0	2.41 ±0.04	1.48 ±0.06
100	0	0	0	0	0	0	2.36 ±0.05	1.47 ±0.01
120	0	0	0	0	0	0	2.35 ±0.02	1.42 ±0.01

Infected dose of *L. monocytogenes*: 1000 cells per 1 m*l*; $n = 3$.

SE: standard error.

Table 2. Dynamics of *Listeria monocytogenes* reproductive forms in frozen **filtered** marine water at different temperatures.

Exposure time (days)	Log CFU/m*l*±SE							
	Strain **2M**		Strain **10CN**		Strain **P**		Strain **1106**	
	−20~−22°C	−4~−6°C	−20~−22°C	−4~−6°C	−20~−22°C	−4~−6°C	−20~−22°C	−4~−6°C
1	2.83 ±0.18	3.33 ±0.20	3.99 ±0.21	3.32 ±0.21	3.47 ±0.22	3.30 ±0.17	3.84 ±0.24	3.37 ±0.21
2	3.86 ±0.23	2.97 ±0.09	3.74 ±0.18	3.36 ±0.22	3.31 ±0.19	3.07 ±0.20	3.21 ±0.19	3.42 ±0.23
3	3.56 ±0.19	2.91 ±0.16	3.10 ±0.19	3.07 ±0.09	3.26 ±0.07	2.89 ±0.14	2.66 ±0.20	3.39 ±0.12
7	3.23 ±0.14	2.09 ±0.11	2.38 ±0.05	1.78 ±0.07	3.1 ±0.06	2.64 ±0.12	2.66 ±0.05	2.62 ±0.06
14	3.13 ±0.12	1.79 ±0.06	2.05 ±0.06	1.57 ±0.09	1.40 ±0.05	2.15 ±0.07	2.64 ±0.04	2.60 ±0.09
30	2.81 ±0.14	0.70 ±0.01	1.90 ±0.02	1.28 ±0.05	1.40 ±0.03	1.62 ±0.05	2.65 ±0.06	1.52 ±0.02
50	1.31 ±0.02	0	1.89 ±0.03	1.20 ±0.06	1.32 ±0.04	0.93 ±0.03	2.61 ±0.13	2.51 ±0.02
60	0	0	1.81 ±0.01	0.82 ±0.06	1.20 ±0.01	0	2.58 ±0.04	2.48 ±0.06
70	0	0	1.20 ±0.02	0.80 ±0.05	0.20 ±0.01	0	2.60 ±0.15	2.47 ±0.11
90	0	0	0.92 ±0.01	0.34 ±0.02	0	0	2.59 ±0.05	2.45 ±0.02
100	0	0	0.31 ±0.02	0	0	0	2.62 ±0.02	2.43 ±0.01
120	0	0	0	0	0	0	2.61 ±0.12	2.42 ±0.10

Infected dose of *L. monocytogenes*: 1000 cells per 1 m*l*; $n = 3$.
SE: Standard error.

temperature conditions were analyzed (Table 2). This experiment shows that the absence of attendant microflora prolonged the reproductive period of *Listeria* strains exposed at −20 to −22°C for at least 10 days. This phenomena was not observed at −4 to −6°C. Only a small increase in the reproductive potential was confirmed among the majority of strains in comparison to strains frozen in unfiltered seawater under the same temperature conditions.

Thus, our experimental data confirm that the duration of the reproductive period of *L. monocytogenes* in sea ice depends on the biological peculiarities of the particular strain and on the temperature regime. Colder temperatures promote longer preservation of *L. monocytogenes* reproduction features in sea ice.

Conclusions

The contamination of sea ice by *Listeria monocytogenes* bacteria in the western part of the Sea of Okhotsk has been verified for the first time. The Amur River outflow is

likely to be the main carrier of *Listeria* to the sea.

Laboratory experiments proved that sea ice is a specific reservoir for *L. monocytogenes*, in which the bacteria are viable during cold periods. Ice rafted migration of *L. monocytogenes* considerably increases its natural habitat area and produces preconditions unfavorable for listeriosis infection in the entire Sea of Okhotsk region.

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