

**EXTRACTION, CHARACTERISATION AND
METAL BIOSORPTION OF
EXTRACELLULAR POLYSACCHARIDES
FROM ACTIVATED SLUDGE.**

by

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APPROVED FOR FINAL SUBMISSION

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ABSTRACT

Waste activated sludge is a biological adsorbent whose potential to remove metals from solution and effluent has been demonstrated. Extracellular polysaccharides (EPS) as components of activated sludge are thought to contribute to activated sludge metal biosorption. During the present study characterisation and determination of the metal biosorptive capabilities of domestic and industrial extracellular polysaccharides (EPS) revealed similarities both in terms of chemical composition and metal adsorption potential. Extracellular polysaccharides were extracted from activated sludge, obtained from domestic and industrial sludge treatment plants, using chemical techniques which involved sodium hydroxide extraction and solvent precipitation. A purification technique, which involved precipitation of protein with chloroform and removal of nucleic acids was developed. To assess the efficiency of the purification method, the ratio of extracted polysaccharide to the amount of protein present was determined. This provided an indication of the magnitude of EPS extracted in relation to the degree of cellular disruption. The type of activated sludge being treated was shown to be of particular importance. The quantity of EPS present in the original sample was found to be higher in domestic sludge than in industrial sludge. Purified EPS was fractionated in a column of DEAE-Sephacrose CL-6B using stepwise pH gradient elution. Molecular weight distribution was conducted on a column of Sepharose CL-4B. Component monosaccharides were identified by paper chromatography. Monomers identified were glucose, fructose, glucuronic acid and galactosamine. Ion-exchange chromatography results demonstrated the presence of a number of different polysaccharide fractions while gel filtration results indicated a wide molecular weight distribution range of EPS from both domestic and industrial activated sludge. This indicated potential for variety in the EPS content of the activated sludge. Metal adsorption studies were conducted to determine the capabilities of EPS to adsorb metals

from solution. Binding of metals to binding sites were found to be dependent on chemical composition and monomer composition of EPS. Components of EPS thought to be responsible for adsorption such as glucuronic acid and amino sugars were found to be more abundant in domestic sludge EPS. Overall, domestic sludge EPS adsorbed larger average percentages of metal-ions from solution than industrial sludge EPS with the following order of adsorption efficiency :

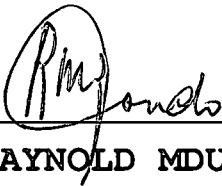
domestic EPS - $\text{Cr}^{6+} > \text{Cr}^{3+} > \text{Ni}^{2+} > \text{Cu}^{2+} > \text{Zn}^{2+} > \text{Cd}^{2+}$ and

industrial EPS - $\text{Cr}^{3+} > \text{Cr}^{6+} > \text{Cu}^{2+} > \text{Zn}^{2+} > \text{Ni}^{2+} > \text{Cd}^{2+}$.

The results of Ni^{2+} - Cr^{6+} biosorption in a binary mixture indicated synergistic interactions that result in an increased adsorption of these metals in a binary system. Therefore EPS as component of the activated sludge plays a major role in the adsorption of metals by the sludge.

DECLARATION

I declare that this dissertation is my own work. It is submitted for the degree of Master's in Technology : Biotechnology at Technikon Natal, Durban. It has not been submitted before for any degree at any other tertiary institution.

A handwritten signature in cursive script, appearing to read 'Raynold Zondo', is written over a horizontal line.

RAYNOLD MDUDUZI ZONDO

DEDICATION

To my parents

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LIST OF ABBREVIATIONS

BSA	-	Bovine serum albumin
BET	-	Brunauer, Emmet and Teller
BOD	-	Biochemical Oxygen Demand
COD	-	Chemical Oxygen Demand
EPS	-	Extracellular polysaccharide
ECP	-	Extracellular polymer
rpm	-	revolutions per minute
TFA	-	Triflouroacetic acid
Ni	-	Nickel
Zn	-	Zinc
Cu	-	Copper
Cd	-	Cadmium
Cr	-	Chromium

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CHAPTER ONE

1.0 GENERAL INTRODUCTION

As a result of a vigorous development of productive capacity and the expansion of the South African economy, volumes of industrial effluents have continued to increase, bringing new problems and challenges to wastewater treatment plants. The expansion of formal and informal settlements and the introduction of piped water system supplying cities and municipal districts has contributed to the problems. Wastewater networks have been an acceptable manner in transporting effluents, thereby eliminating a source of infection experienced over the years. However, adverse effects on the environment at large, brought about by unpleasant odours, metal laden estuaries and fish kills are some of the serious effects being experienced (Horneglasson and Partners, 1989).

In South Africa the motivation for successful purification of industrial and domestic wastewater is an urgent requirement for preservation of our limited resources. South Africans are at a significant environmental, social and health risk as a result of uncontrolled waste generation and disposal. Good waste management is important for sustainable use of the country's limited natural resources and for adequate and sustainable protection of water, air and soil on which all citizens depend. Water has been identified

as South Africa's most limiting natural resource and every effort must be made to conserve it.

The most significant sources of water pollution are large concentrated discharges from wastewater and industrial effluent treatment works (Eckenfelder and Musterman, 1995). Numerous industrial activities produce liquid wastes containing pollutants such as metals, phosphates, fertilisers, etc. Many industrial uses of metals involve the discharge of metal-laden effluents to the sewage system (Eckenfelder and Musterman, 1995).

One of the methods which can be used to achieve this is to increase water reuse. Before reuse, however, water must be treated to an acceptable standard for either direct reuse, or discharge into the aquatic environment without seriously affecting the environment (Horneglasson and Partners, 1989).

1.1. HEAVY METALS

Heavy metals have been the cause of particular environmental concern. These are generally accepted to be chromium, manganese, iron, cobalt, copper, zinc, molybdenum, silver, mercury, cadmium and nickel (Brown and Lester, 1979). They contribute to effluent aquatic toxicity and thereby limit the options for ultimate solids disposal. Heavy metal pollutants from industrial processes such as electroplating and mining water effluents are threatening South

Africa's limited drinking water supplies. These heavy metals are an important group among the pollutants present in wastewaters because of the impact they have on the environment. This has led to the formulation of guidelines for the regulation of the use of sewage sludges contaminated with metals that are produced as a result of metal removal during wastewater treatment (Kasan, 1993).

Legislation governing protection of the environment is becoming stricter. It is important, therefore, that high concentration of metals in raw sewage entering wastewater treatment plants are removed, since the discharge of metal-laden effluents to water courses may produce toxic effects in the aquatic environment (Brown and Lester, 1979). That proportion of the heavy metal input which is not removed during activated sludge process is usually discharged directly via the effluent to surface watercourses.

The disposal of solid and liquid waste products resulting from industrial processes is receiving increasing attention on a global basis. The negative impact of these metals and their accumulation through food chains has prompted research into the development of alternate wastewater purification technology. To contribute to these developments, the environmental biotechnologist is faced with the special challenge of developing appropriate methods for the treatment and/or reuse of waste products prior to discharge into the environment (Kasan, 1993).

Bioremediation has been investigated for its ability to remove metals from these wastewaters in a cost effective and environmental friendly manner (Volesky, 1990; Bux and Kasan, 1996). The potential recovery of valuable metals is an attractive consideration in the bioremediation process and may enhance its economic viability (Tsezos, 1990). Initial investigations have focused on the ability of different microorganisms to bioaccumulate specific metals and the parameters which influence the metal removal capacities (Brady and Duncan, 1994). Mechanisms of uptake have been studied by Brady and Duncan (1994) to gain understanding and allow manipulation of metal bioaccumulation.

The capacity of microorganisms to concentrate heavy metals has been well studied (Brown and Lester, 1982; Kasan, 1993; Swalaha, 1993). The term "biosorption" is defined as a process in which solids of natural origin, e.g. microorganisms, alive or dead, or their derivatives, are employed for sequestration of heavy metals from an aqueous environment. The mechanism of uptake can be due to physical sorption, chemical complexation with cell surface groups or bioaccumulation (Brown and Lester, 1979). Biosorption depends not only on the chemical composition of the cell or its components such as the cell wall but also on the external physico-chemical factors and the solution chemistry of the metal.

There is currently a trend towards biological methods for remediation of metal contamination with the use of existing

biological material of specially developed system. These methods should be cost effective and environmentally friendly.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 INTRODUCTION

In recent years wastewater treatment processes have assumed an important status in the hydrological cycle. Accepted terminology for all the processes which handle domestic sewage and mixed industrial-domestic wastewater has gradually evolved from "wastewater disposal" through "wastewater treatment" to "wastewater reclamation", reflecting a change in attitudes towards wastewater as a vital resource (Chong and Volesky, 1995).

Wastewater, which comprises sewage and/or industrial effluents, contains many substances some of which are in solid form whilst others are dissolved in the liquid. On arrival at the treatment works the wastewater is subjected to a number of treatment processes prior to being discharged. The treatment processes are designed to ensure that the effluent being discharged to the river/watercourses complies with prescribed standards set by the Department of Water Affairs and Forestry (City of Durban, 1994).

Heavy metals in waters are of concern because of their known toxicity and harmful effects on biological treatment processes. In

treatment process, generally only 5 to 20% removal occurs in the primary sedimentation basin, while 10 to 80% removal occurs in the activated sludge aeration tank. The activated sludge process is the most widely used secondary wastewater treatment process (Jenkins *et al.*, 1993). The role of bacterial extracellular polymer in relation to metal removal in the activated sludge process has been investigated (Brown and Lester, 1979). Many workers who have investigated extracellular polymer production in activated sludges found that relatively large amounts produced are polysaccharides (Forster, 1976; Brown and Lester, 1979; Sterrit and Lester, 1982).

2.2 ACTIVATED SLUDGE PROCESS

The activated sludge process is currently the most popular method for treatment and disposal of wastewaters emanating from domestic and industrial sources. The principle of activated sludge is that, in a reactor, a community of microorganisms is constantly supplied with organic matter and oxygen (Jenkins *et al.*, 1993). Microorganisms consume the organic matter and transform it by means of aerobic metabolism, partly into new microbial biomass and partly into CO₂, H₂O and minerals. The flow of water brings about constant wash-out of microorganisms from the reactor to the settler. Here, the microorganisms which grow in flocs are retained and then removed with the underflow. Part of the sludge is then recycled to

provide biomass to treat new influent. The surplus amount is discarded. This product of the activated sludge process is waste activated sludge and is largely comprised of biomass produced during the process of bioconversion of wastewaters. Biomass is comprised of bacteria, fungi, algae and protozoa (Bux *et al.*, 1994). The wastewater usually contains heavy metals such as Cu, Zn, Ni and Cd which originate predominantly from industrial discharges. Activated sludges have shown the ability to adsorb these metal cationic species from solution (Kasan, 1993; Swalaha, 1993).

Activated sludge may be defined as a flocculent microbial mass produced when sewage is continuously aerated (Kasan, 1988). Activated sludge treatment is widely utilised for the removal of organic pollutants from wastewater which are converted to biomass during vigorous aeration. It is an efficient biological method for the stabilisation of domestic and industrial wastewater.

A consequence of wastewater treatment by the activated sludge process is the production of substantial quantities of waste activated sludge. Biomass produced during the activated sludge process is allowed to flocculate after aeration, and settle-out, removing most of the biological and chemical oxygen demand from solution. Waste sludges produced by this process can be used as fertilisers due to the high nitrate and phosphate content. However, because sludges are often contaminated with metal ions,

they cannot be used as fertilisers due to the possible contamination of groundwater and toxic effects to plants and animals.

There is currently a trend towards biological methods for remediation of metal contamination with the use of existing biological material. Waste activated sludge is a biological adsorbent whose potential to remove metals from solution and effluent has previously been demonstrated (Kasan 1993). Trials have been conducted using activated sludge to determine its feasibility for adsorption of metals from industrial effluents (Kasan and Baecker, 1990; Swalaha and Kasan, 1991; Swalaha and Kasan, 1992; Kasan, 1993; Bux *et al.*, 1996). Sludges have been shown to possess potential for biosorption of metals. Extracellular polymer components in activated sludge have also been reported to remove metal ions from solution (Brown and Lester, 1979; Brown and Lester, 1982; Swalaha 1993).

2.3 METAL BIOSORPTION BY ACTIVATED SLUDGE

Kasan (1993) showed that metal ions in the activated sludge treatment process are toxic to bacteria and inhibit organic matter reduction. Autotrophic nitrifying bacteria are more inhibited by metal ions than heterotrophic bacteria. Historically there has

been little concern for the presence of these heavy metals in the wastewater as long as they were at subtoxic level. But increasingly, stringent effluent regulations have resulted in a need to assess the interaction and removal efficiency of heavy metals within the activated sludge process (Kasan, 1993).

Many industrial uses of metals, for example, metal plating, tanneries, industrial processes utilising metals as catalysts, contribute to the discharge of metal-laden effluents to the sewage system. High concentrations of metals in final effluents from wastewater treatment plants would result in increased metal concentration in lowland rivers from which some water supplies are obtained.

TABLE 2.1. Major sources of heavy metals in activated sludge. (Umgeni Water, 1997).

Metal	Major Source
Zinc	Domestic, electroplating and chemical industries
Copper	Electroplating, chemical industries
Nickel	Motor industries, printing and chemical
Cadmium	Electroplating, pigment and chemical
Chromium	Electroplating, tanning and dye industries.

The main problem faced by wastewater treatment plants is disposal of this metal contaminated waste sludge. Several methods of

disposal are currently being practised such as disposal to landfills or alternatively spreading onto agricultural land, but because of the presence of heavy metals in sludge these methods are not feasible. It is therefore important that high concentrations of metals in raw sewage entering a wastewater treatment plant be removed during the treatment process or before the effluent reaches wastewater treatment plants.

Heavy metal removal in the activated sludge process has been widely studied. Sterrit *et al.*, (1981) demonstrated the ability of activated sludge to adsorb heavy metals in solution. He attributed this ability to two distinct mechanisms of metal removal in activated sludge, precipitation and adsorption of predominantly soluble metals by the biomass. Precipitation results from the formation of insoluble hydroxides, carbonates and orthophosphates (Stoveland and Lester, 1980).

Stoveland and Lester (1980) also highlighted the fact that the removal of heavy metals during the sludge process occurs in two stages. First by primary settling of insoluble metals and secondly, by the adsorption of dissolved metals to the biological floc, which is then removed by settling. However, they reported that primary treatment has little effect on the concentrations of dissolved metals in the sewerage.

Physical, chemical and biological factors influence heavy metal

removal in the activated sludge process. The principal biological factor is the concentration of bacterial extracellular polymers found in wastewater bacteria. Dugan and Pickrum (1972) showed that eight species of floc-forming bacteria isolated from wastewater adsorbed metal ions. Hutchins *et al.*, (1986) illustrated an active process of metal accumulation in bacteria while McKinney (1952) related flocculation to the surface properties of the microbes involved.

Many of the proposed mechanisms of bioflocculation in activated sludge involve the formation of extracellular polymers (McKinney and Horwood, 1952; McKinney and Weichlein, 1953). The accumulation of polymeric material often increase with increasing culture age and was shown to be directly correlated with the flocculation. Divalent cations are considered to be important bridging agents between negatively charged exopolymers and bacteria (McKinney, 1952). The mechanism of heavy metal adsorption to the sludge surface occur in different bacteria when extracellular gels are produced, but the metal-polymer interaction is not yet fully understood.

2.4 COMPOSITION OF FLOCS

One of the phases involved in development of a high quality

effluent during the treatment process, is the flocculation of microorganisms into settleable mass so that a clear low BOD end product may be obtained. The purpose of flocculation is to aggregate as many of the precipitated primary particles into separable flocs as possible and to prepare the flocs to be separated as early as possible. Bioflocculation is an essential event in activated sludge wastewater treatment. Activated sludge microorganisms produce soluble polymers which are implicated in floc formation (Brown and Lester, 1979).

Flocculation can be defined as the aggregation of suspended bacterial cells after growth of the cells has occurred. Several theories of flocculation have been proposed for bacteria in pure cultures and in activated sludge (Busch and Stumm, 1968; Friedman *et al.*, 1968). Most of theories which have been formulated to explain flocculation in activated sludge propose that bacterial EPS play a key role in the process (Busch and Stumm, 1968; Friedman *et al.*, 1968)

Activated sludge flocs are composed of a wide variety of bacteria, fungi, algae, and other microorganisms in the presence of dissolved oxygen. Dias and Bhat (1964) isolated more than 300 microbial strains from activated sludge and found *Zoogloea* and *Comamonas* species to be predominant. Some of these microorganisms have been shown to be involved in bioflocculation. The major component of floc appears to consist of heterotrophic bacteria that include such

genera as *Pseudomonas* sp., *Achromobacter* sp., *Flavobacterium* sp., *Alcaligenes* sp., *Citrobacter* sp., and *Zoogloea* sp. (Blackbeard, et al 1986; Jenkins et al., 1993; Bux et al., 1996). In addition flocs contain inorganic and organic material as well as extracellular polymeric substances. Cheng et al., (1975) described the floc as formed by the polysaccharides, protein and nucleic acids which provides functional groups that act as binding sites for metals and constitute a matrix in which cells are embedded and by which they adhere to one another.

The electrical surface charge of microorganisms is considered to be an important factor in bioflocculation, and the formation of capsules or slime also contributes to this phenomenon. It has been established by Brown and Lester (1979) that capsules and slime consist of polysaccharides with varying contents of acetyl and amino groups. These may contribute to the net surface charges of the polysaccharides, thereby playing a role in the flocculation process (Ikeda et al., 1982). McKinney (1955) postulated that the envelopment of the cell wall by the polysaccharide material serves to displace the attractive forces between similar cells. Wilkinson (1958) followed this investigation describing the various types of microbially produced polysaccharides and their characteristics.

2.5 FLOC FORMATION

The process of bioflocculation has been proposed by McKinney (1952) to be, in one way or another, related to surface properties of the microbes involved. Steiner *et al.*, (1976) detected sensitive components in sludge which contribute to overall charge carried by the surface. Their conclusion was that the sludge matrix is composed of two polymeric species which contribute to the negative charge carried by the sludge.

A mechanism of flocculation has been proposed based on high molecular weight extracellular polymers which bridge bacterial cells, resulting in a three dimensional matrix or floc (Pavoni *et al.*, 1972; Tenny and Verhoff, 1973). Tenny and Verhoff (1973) observed that the amount of flocculation in a bacterial culture was related to the amount of extracellular polymers present.

Forster (1976) suggested that these polymers were polysaccharide in nature and were composed of neutral sugars and uronic acids. Previous work has demonstrated the presence of proteins and nucleic acids in the polymers of activated sludge (Pavoni *et al.*, 1972). Forster (1971) expanded this theory by proposing that two different types of polymeric bonding are involved in flocculation. Ionic bond as a result of the acidity of extracellular polymers may result in flocculation and neutral polysaccharides may also cause

flocculation by hydrogen bonding through regions of electron density.

Treatment of polymer with a suitable exopolymer-degrading enzyme have been shown to cause deflocculation (Tago and Aida, 1977). But some floc containing heteropolysaccharides, which are more biologically stable, resist the effect of these deflocculation enzyme.

Pavoni *et al.*, (1972) found a correlation between exocellular polymer accumulation and microbial agglutination. They observed an increase in exocellular polymer:microorganisms ratios during agglutination. Therefore, the mechanism for bioflocculation can be seen to be resulting from interaction of high-molecular weight exocellular polymers, which have sufficiently accumulated at the microbial surface during endogenous growth. Bioflocculation can thus be viewed in terms of surface coverage relationships. For bioflocculation mechanism to be clearly understood, it is important to understand the compositional make up of naturally produced EPS thought to be responsible for microbial aggregation.

2.6 ROLE OF EXTRACELLULAR POLYSACCHARIDES IN BIOFLOCCULATION

Bioflocculation is important to enable efficient operation of activated sludge wastewater treatment processes (Morgan *et al.*, 1990). Extracellular polymers are believed to play an important role in bioflocculation in biological wastewater treatment processes. Tenny and Stunn (1965) proposed that polymers exposed on the microbial surface may act to absorb and form bridges between cell surfaces and therefore initiate floc formation.

The role of extracellular polysaccharides in relation to bioflocculation and their effect on sludge physiochemical characteristics, such as settleability, dewatering, floc strength and charge, are incompletely understood (Morgan *et al.*, 1990). Most of the proposed mechanisms for bioflocculation are based on the complex interaction between these high molecular weight polymers which bond electrostatically and physically to microbial surfaces.

There has been research done on sludge surface polymers which has focused upon the polysaccharide moiety which is produced extracellularly by bacteria and occurs as both a discrete slime and an attached capsule (Sutherland, 1972).

2.7 BACTERIAL EXTRACELLULAR POLYMERS

Pavoni *et al.*, (1972) found that the activated sludge extracellular polymer has carbohydrate as its dominant component. However Morgan *et al.*, (1990) found that the ratio between protein and carbohydrate components is variable and is dependent on the extraction method used and type of waste being treated. Vallom and McLoughlin, (1984) reported that the protein fraction was dominant in extracellular polymer extracted from activated sludge that had been acclimatised to a feed containing a high concentration of Bovine Serum Albumin (BSA).

It has been demonstrated that purified extracellular polymer from bacterial cultures may take up and concentrate metal ions from solution (Brown and Lester, 1980). The environmentally important properties of extracellular polymers are determined by the characteristics of the individual carbohydrate residues, the net charge of polymers and the degree of branching.

According to Wilkinson (1958) the bacterial polysaccharides make up a group of polymers in which the structural variations is almost unlimited, and unusual sugars are often components of these polymers. The most extracellular polymeric substances found in aquatic environments are the capsules and slime (Geesey, 1978).

Geesey (1978) described capsules as extracellular polymers tightly associated with the cell surface and slime being more loosely associated with the cell surface. Capsules differ widely in composition among bacterial species and consist of linear polymers of polysaccharides or amino acid repeating units (Sutherland, 1972). Exopolysaccharides usually possess a repeating sequence of two or six sugar subunits (Ehrlich and Brierley, 1991). The polysaccharide varieties may or may not contain monosaccharide side chains and many sugars may also contain a reactive carboxyl and amino groups in a side chain.

It has been postulated that because capsules may contain anionic groups such as carboxyl groups (Sutherland, 1985), it is not surprising that they may be capable of binding metals (McLean and Berveridge, 1988). According to Brown and Lester, (1980) bacterial extracellular polysaccharides in activated sludge maintain extensive complexing capacity for heavy metals. Polysaccharides also contain an abundance of hydroxyl groups which tend to interact with metal ions. At neutral pH, the partially ionized carboxyl groups are available to interact with positively charged metal ions.

Ikeda et al., (1982) found that purified polysaccharides from *Zoogloea* sp. flocculated in the presence of several metals. Since binding of metals to this polymer promoted flocculation, the reaction had been proposed by Dugan (1970) as a means of removing

metal ions in a concentrated form from acid mine water.

The ways in which heavy metal ions and activated sludge flocs interact are important to several aspects of wastewater treatment (Brown and Lester, 1979). There is presently a great need to identify polymers that demonstrate high selectivity for metal ions such as Cu, Ni, Zn and Cr which are common in industrial effluents.

2.8 STRUCTURE OF EXTRACELLULAR POLYSACCHARIDES

Polysaccharides are polymers of monosaccharide units joined together by glycosidic bonds which are formed by the elimination of the elements of water between the hemiacetal hydroxyl groups of one residue and a primary or secondary hydroxyl group of an adjacent residue (Wilkinson, 1958).

Polysaccharides may consist of a small or a large number of residues and in structure may be linear, branched or occasionally cyclic. These polymers may be homopolysaccharides or heteropolysaccharides. In living cells, polysaccharides may be combined covalently with members of other classes of compounds notably proteins and lipids. Many of these combinations as well as free polysaccharides have important biological functions and the group has become as complex carbohydrates (Sutherland, 1972).

Extracellular polymers can be described as being high molecular weight compounds produced by microorganisms under certain environmental conditions (Morgan *et al.*, 1990). The composition of bacterial extracellular polysaccharide has been extensively reviewed by Sutherland (1985) who suggested that polymers produced by certain bacterial species are almost composed entirely of neutral sugars and a limited number of uronic acid.

Horan and Eccles (1986) have shown that this is true for activated sludge polymers. Analysis of the monomer composition of five purified sludge polysaccharides from different treatment works showed that five monomers, namely: glucose, galactose, mannose, glucuronic acid and galacturonic acid, were the most abundant by percentage weight in all samples. Some polysaccharides contain amino sugars or sugars with amine or amide - linked functional groups (Henry and Corale, 1990).

2.9 BINDING OF METALS TO EXTRACELLULAR POLYSACCHARIDES

Bacterial extracellular polymers have been shown to be involved in flocculation in bacterial cultures and in the adsorption of metal ions from solution (Brown and Lester, 1979). These polymers extracted from activated sludge are polysaccharide in nature.

The evidence presented by Brown and Lester (1979) suggests that

different metal adsorption sites exist on neutral polysaccharides and anionic polysaccharides. According to Foster (1985) metal binding sites can be characterised in terms of the number of binding sites available, the type of sites and the ability of the molecule to form complexes. Metal ions of different valencies or with different charges may also bind at different sites.

Dugan *et al.*, (1969) observed that many bacterial extracellular polymers bind water to a greater extent. They proposed that cations may exchange with water.

Several possible mechanisms required to facilitate metal removal have been proposed such as :

- (a) the binding of soluble metal to extracellular polymer,
- (b) accumulation of soluble metals by the cell, and
- (c) physical trapping of precipitated metals in the sludge floc matrix (Brown and Lester, 1979). Parameters that affect the process are operating pH and floc size (Eckenfelder and Musterman, 1995).

Rendelman (1978) has defined two major types of metal-polysaccharide complexes :

- (a) Complexes with anionic polysaccharides, and

(b) Metal complexes with neutral polysaccharides.

However, Rendelman (1978) found that, in aqueous media, neutral polysaccharides have little affinity for cations with little ability to polarise a donor atom. Even at low concentration, anionic polysaccharides have a strong affinity for metal "counterions".

Rendelman (1978) also proposed that in complexes where ligands are uncharged, the bonding attraction is between the cation and the dipole of the donor molecule. When the ligand has charged groups, the bonding is largely ionic with varying amounts of covalent character.

Where polymers are anionic polyelectrolytes, for example proteins and carbohydrates with uronic acid residues, carboxyl groups can be the metal binding sites (Brown and Lester, 1979). Polysaccharides also contain an abundance of OH groups which tend to interact with metal ions. The electronegative oxygen atoms of hydroxyl groups are likely to participate in metal interactions with anionic or neutral polysaccharides (Henry and Corale, 1990)

Although EPS has frequently been cited as an important factor that determines heavy metal uptake capacity of the activated sludge, (Cheng et al., 1975; Sterrit and Lester, 1980; Nelson et al., 1981), studies on the characterisation of metal binding properties of sludge EPS are scant.

2.10 HYPOTHESIS

That extracellular polysaccharides from activated sludge are high molecular weight heteropolysaccharides which contain sites that are capable of adsorbing large quantities of metal ions from solution. Many of these polysaccharides have negatively charged groups and positively charged groups which have metal complexation capability.

2.11 AIMS

- a) To develop an extraction technique which results in extracellular polysaccharides substantially free of proteins and nucleic acids.
- b) To biochemically characterise extracellular polysaccharides extracted from activated sludge.
- c) To determine metal-biosorptive capabilities of extracellular polysaccharides.
- d) To determine functional groups responsible for metal biosorption by extracellular polysaccharides.

2.12 OBJECTIVES

- a) Extraction of extracellular polysaccharides (EPS) from activated sludge.
- b) Purification of the extracted activated sludge EPS.
- c) Qualitative analysis of the purified EPS.
- d) Determination of the polysaccharide monomer composition and molecular weight distribution of EPS.
- e) Determination of the chemical composition of EPS.
- f) Determination of the metal adsorptive capabilities of EPS.
- g) Identification of the correlation, if any, between chemical composition of EPS and their metal adsorptive capabilities.
- h) Application of EPS as metal adsorbents and the contribution of charge and molecular weight on metal adsorbent capabilities of EPS.
- i) Determination of selectivity of EPS in metal uptake.

CHAPTER THREE

3.0 EXTRACTION AND PURIFICATION OF EXTRACELLULAR POLYSACCHARIDES FROM ACTIVATED SLUDGE

3.1 INTRODUCTION

In pure cultures obtained from wastewater, up to 40% and 50% (cell dry weight) of bacteria such as *Zoogloea ramigera* 115 and *Klebsiella aerogenes* respectively, may consist of extracellular polymers (Parson and Dugan, 1971; Rudd et al., 1984b). These bacteria have also been isolated from activated sludge and similar results have been confirmed (Brown and Lester, 1979). Exopolymeric materials such as polysaccharides, polyamino acids, polynucleic acids, and their complex polymers can usually be found in activated sludge (Harris and Mitchell, 1973). According to Brown and Lester (1980), these are part of the floc-polymer matrix and are mainly composed of polysaccharides.

Numerous studies have been devoted to the extracellular polymer matrices in an attempt to pinpoint their role in activated sludge flocculation (Brown and Lester, 1979; Vallom and McLoughlin, 1984; Forster, 1985; Horan and Eccles, 1986). The polysaccharides found

within the extracellular matrices have not been studied in great detail. The main problem in achieving greater elucidation of these compounds is contamination during extraction of EPS with proteinaceous and lipid material. In fact, although polysaccharides predominate and represent up to 65% of extracellular materials (Horan and Eccles, 1986), other substances such as proteins, nucleic acids and lipids are also present (Goodwin and Forster, 1985).

The isolation of these polysaccharides in a purified form is an important step in their chemical and physical characterisation. Almost all information concerning the chemical composition of EPS has been obtained through analysis made directly on activated sludge samples (Lazaremo and Manem, 1995). Information on the nature of EPS may be obtainable only after successful extraction and purification has been achieved. If the material that is to be studied is associated with particulate matter, the first step in the purification procedure is extraction of material with a suitable solvent.

During extraction one compound may be dissolved and hence extracted from its original environment while another is left behind. It is therefore imperative that since EPS is found in the activated sludge with lipids, proteins and nucleic acids, there should be separation of these polymeric materials from EPS. This can lead to a detailed structural investigation of EPS after the purity of the

product has been established.

Extraction of EPS from activated sludge has been conducted by different procedures that are sometimes complex. Attempts that have been made to extract and quantify sludge biopolymers include steaming (Wallen and Davis, 1972) and ethanolic extraction (Foster and Clarke, 1985). Brown and Lester (1980) compared five bacterial extracellular polymer extraction methods. These were ultracentrifugation, autoclaving, disodium ethylenediamine acetic acid (EDTA), NaOH and thermal extraction methods. Their findings show that these techniques do not result in a highly purified EPS.

Other methods that have been developed in the laboratory involve several basic steps such as fractional precipitation of EPS by adding acetone (Novak and Haugan, 1981), ethanol (Novak and Haugan, 1981), propan-2-ol (Horan and Eccles, 1986) and methanol (Friedman *et al.*, 1968). These steps are unsuitable since they result in coprecipitation of other polymeric materials such as proteins. Horan and Eccles (1986) overcame this difficulty by including nuclease and protease treatment of samples in their purification procedure. These materials are then extracted by chloroform:amyl alcohol precipitation. After all these steps purity can be tested by comparing the ratio between sugar and protein in each purification step. Most of the above methods have a very low efficiency in terms of selective EPS extraction and promote cellular lysis or intracellular material loss which can distort the

results (Azaredo et al., 1997).

Therefore, as part of a study to quantify and characterise polysaccharides from a variety of activated sludge treatment plants, the aim of the following part of research was to develop an extraction and purification method characterized by high efficiency and minimal effect on cell lysis. This will result in the purification of the extracellular polysaccharides from domestic and industrial sludge and the development of a purification procedure which results in a high quality EPS. A high degree of purity will facilitate studies of the relationship between chemical structure and biological function.

3.2 HYPOTHESIS

Domestic and industrial activated sludges contain extracellular polymers the bulk of which are polysaccharides in nature. These polysaccharides can be isolated and purified.

3.3 AIM

To develop an extraction technique which results in extracellular polysaccharides substantially free of proteins and nucleic acids.

3.4 OBJECTIVES

- a) To extract and purify the extracellular polysaccharides from domestic and industrial activated sludge.
- b) To determine the amount of EPS present in both type of sludges.
- c) To determine the differences in composition and purity between domestic sludge EPS and industrial sludge EPS.

3.5 MATERIALS AND METHODS

3.5.1 Sample Collection

Activated sludge samples were obtained from the following Wastewater Plants operated in the Durban Metro Region : Phoenix; Kwamashu; New Germany; Amanzimtoti; Umbilo and Southern Works. Sludge was stored at 4 °C. Preliminary extractions were performed on the same day as sampling.

3.5.2 Extraction and purification of polysaccharides

Activated sludge samples were dissolved in 1 volume of 2 M NaOH with continuous stirring at 4 °C for 24 h. Samples were centrifuged at 4000 rpm (Beckman J6-MC model) for 15 min. The resultant pellet was discarded. Supernatants were filtered through pre-dried, pre-weighed glass-fibre filter paper. Extracted polymers were precipitated with stepwise addition of 1 vol acetone:ethanol (3:1) while continuously stirring at 4 °C for 24 hours. This solution was centrifuged at 4000 rpm for 10 min at 4 °C to recover all polymers in suspension. The precipitate was dissolved in 100 mM Tris/HCl buffer (pH 7).

Purification of EPS was done by addition of 1 volume of chloroform to the solution kept in an orbital shaker rotating at 150 rpm for 3 h. Sample were centrifuged at 4200 rpm for 15 min. A gel-like protein layer was separated from the supernatant containing EPS.

Extracellular polysaccharides were visualized as flocculent precipitates after 24 h at 4 °C. Further purification of EPS was conducted by incubating the sample with protease (50 Units) overnight at 37 °C. Nucleic acids were removed using 200 KU DNase and 400 KU RNase at 25 °C for 24 h. Protein residues were subsequently removed by addition of 0.1 volume of chloroform:amyl alcohol (10:1). The suspension was centrifuged for 30 min at 4200 rpm at 25 °C. A clear supernatant was carefully decanted and dialysed against 3 litres of deionised water. The dialysed sample was subsequently lyophilised. A brown sponge-like powder, industrial EPS, and a light-brown sponge-like powder, domestic EPS, made of tiny fibres were then dissolved in a Tris/HCl buffer and this represented purified polysaccharides.

3.5.3 Biochemical analysis of extracted extracellular polysaccharides.

Total carbohydrates were determined using the phenol/sulphuric acid method of Dubois *et al.*, (1958). The colour was allowed to develop for 30 min before reading the absorbance against the blank at 490 nm. Glucose was used as standard in the range 0-100 mg. ℓ^{-1} . Pentose residues were determined by the Ferric-orcinol assay at 660 nm. Proteins in the extracts were determined using the Folin method described by Lowry *et al.*, (1957). The absorbance of the colour which was developed after 30 min. was read at 750 nm against the blank. A Bovine Serum Albumin (BSA) standard within the range 0-40 mg. ℓ^{-1} was used to determine the concentration of proteins in the crude polymer.

3.6 RESULTS

In order to assess the efficiency of extraction and purification methods, it was necessary to ascertain the amount of extracted carbohydrates and proteins in each step. The ratio of extracted polysaccharides to proteins provides an indication of the magnitude of EPS extraction in relation to the degree of cellular disruption.

Table 3.1 Quantitative determination of total sugars (glucose equivalents) by phenol/sulphuric acid method (Dubois *et al.*, 1958) and total proteins (Lowry *et al.*, 1957) on extract of domestic sludge.

Treatment	Sugar (g.l ⁻¹)	Protein (g.l ⁻¹)	Ratio
NaOH	1.83	1.71	1.07 : 1
Acetone:ethanol	1.51	1.43	1.06 : 1
Chloroform	0.461	0.238	1.94 : 1
Dialysis	0.205	0.184	1.11 : 1

Table 3.2 Quantitative determination of total sugars (glucose equivalents) by phenol/sulphuric acid method (Dubois et al., 1958) and total proteins (Lowry et al., 1957) on extract of industrial sludge.

Treatment	Sugar(g.ℓ ⁻¹)	Protein(g.ℓ ⁻¹)	Ratio
NaOH	0.987	1.59	0.621 : 1
Acetone:ethanol	0.845	1.35	0.626 : 1
Chloroform	0.373	0.115	3.240 : 1
Dialysis	0.268	0.0912	2.940 : 1

The extraction of polymer by dissolving the solution in sodium hydroxide and precipitating it with acetone:ethanol proved to be successful as it decreased the amount of protein present in sample Table 3.1 and Table 3.2). Less than 20% of proteins extracted by NaOH were excluded from supernatant by acetone:ethanol precipitation in both samples. Inclusion of ethanol increased protein exclusion in the precipitate. Although there was a tendency for co-precipitation of the protein with polysaccharides, repetition of the acetone:ethanol precipitation overcame this to some extent. This is confirmed by an increase in ratio of sugar to protein after acetone:ethanol precipitation in industrial sludge samples (Table 3.2).

It is also clear from Table 3.1 and Table 3.2 that the NaOH extraction method may have caused cellular disruption which resulted in large amounts of proteins being released. Brown and Lester (1980) observed that the amount of proteins and nucleic acids present after extraction is indicative of the degree of harshness of the method used. Therefore according to their findings, harshness is directly related to cellular disruption.

Chloroform precipitation removed 83% and 93% of protein from domestic and industrial sludge respectively. Although this step proved to be successful in removal of proteins, the percentage sugar lost in the precipitation was high. It was found that 69% and 62% of sugar present in the original sample was lost in domestic and industrial sludge respectively.

3.7 DISCUSSION

Almost all the information concerning chemical composition of the EPS has been obtained through the analysis made directly on activated sludge samples (Lazaremo and Manem, 1995). Most of these methods have a very low efficiency in terms of selective exopolymer extraction and promote cellular lysis which can distort the results. In the published literature, the ratio between protein, carbohydrates, nucleic acids and lipids in EPS varies, depending on the sample source and the extraction technique. However, as suggested by Ryssov-Nielson (1975), the polysaccharide component present in samples were most dominant. The type of waste being treated was shown to be of particular importance with high amounts of polysaccharides found to be in domestic sludge samples.

Purity was tested by comparing the amount of sugars and proteins left in the sample after each purification step (Table 3.1 and 3.2). Dialysis of the sample against deionised water after chloroform precipitation resulted in the exclusion of amino acids and monomer sugars from solution. This step was important since amino acids are soluble in water and, therefore, could not be excluded from solution by chloroform precipitation.

The degree of purity in the industrial sludge EPS was greater than in the domestic sludge EPS. There was higher protein "contamination" in the purified domestic sludge polysaccharide

(Table 3.1). This may be due to covalent bonding of some proteins to polysaccharides. The prevalence of protein fractions has been demonstrated previously by Forster (1983) and by Swalaha (1993) who quoted a protein:carbohydrates ratio to be 1:3. This result is almost similar to the ratio found for the original industrial sludge sample in the present study (Table 3.2).

The quantity of sugar present in the original sample indicates that more sugars are present in the domestic activated sludge than in the industrial activated sludge. The amount of proteins present also showed that domestic sludge has a high protein content. Although a high protein:sugar ratio was present in the original industrial sludge sample, the purification procedure produced a high quality industrial EPS compared to domestic EPS (Table 3.2). This may be an indication that most of the protein present in industrial polymers are not linked to polysaccharides but are a result of cellular disintegration and/or autolysis.

The inclusion of protease proved to be useful since 83% and 93% of the protein was hydrolysed in domestic and industrial polymer samples respectively and then extracted by centrifugation and dialysis. More than 90% of the protein present in the original samples was extracted. Complete removal of protein proved to be impossible. This may be due in part to the presence of a glycoprotein in which the protein, linked covalently to the polysaccharide moiety could not be precipitated by chloroform.

It is also possible that the protein moiety of polysaccharides undergoes some hydrolysis as a result of the protease treatment (Horan and Eccles, 1986). The increase in quality of the polysaccharide fraction in the supernatant after chloroform precipitation may confirm this fact. The amino acids which may have resulted from protease hydrolysis and remained in the supernatant, were removed by dialysis against deionised water. More than 90% of the protein was removed from polysaccharide solution.

3.8 CONCLUSION

Since 0.205 g.l^{-1} and 0.268 g.l^{-1} of EPS were isolated from domestic and industrial activated sludge respectively, the purification procedure used was successful, although it was not possible to extract proteins completely from EPS. It can therefore be concluded that if this protein contamination is as a result of the harshness of the method used, it is necessary to develop a universal method which will completely eliminate and/or minimise cellular disruption. Chromatographic techniques can be used to determine further characteristics of the purified extracellular polysaccharides. Therefore the next phase of the study was to apply different chromatographic techniques to characterise extracellular polysaccharides.

CHAPTER FOUR

4.0 CHARACTERISATION OF PURIFIED POLYSACCHARIDES FROM ACTIVATED SLUDGE.

4.1 INTRODUCTION

Activated sludge micro-organisms produce soluble polymers which are implicated in bioflocculation (Forster, 1976). These polymers have been shown to be composed of proteins, nucleic acids and polysaccharides (Brown and Lester, 1979). Biopolymers are thought to be influential in determining some sludge characteristics such as surface charge, which is usually negative (Horan and Eccles, 1986). Bux (1996) showed that all sludges investigated around the Durban Metro Area produced an overall net negative charge. They attributed differences in electronegativity of sludges to the chemical nature of the sludge surface influencing the magnitude of surface charge. Stumm and Morgan (1962) and Forster (1971) found the principal ionogenic component of sludge polysaccharide to be glucuronic acid which, at neutral pH, contributes a strong negative charge.

Naturally occurring polysaccharides vary greatly in molecular size ranging from several thousands to millions of daltons (Sutherland,

1972). The molecular structure is diverse but can be divided into three major types: a linear structure, substituted linear structure and a branched structure (Sutherland, 1985). In a linear structure units are joined by the same type of glycosidic linkage to form long linear chains. A substituted linear structure has monosaccharides or short side chains of oligopolysaccharide units attached to the main chain and a branched structure has long side chains attached to the main chain and to the side chain. According to Wilkinson (1958) bacterial polysaccharides make up a group of polymers in which the structural variations is almost unlimited and unusual sugars are often components.

Many of the EPS produced by microorganisms found in activated sludge possess negatively charged organic groups such as carboxylic, aliphatic, aromatic, hydroxyl, sulphate and amino groups, which confer an overall negative charge to sludge floc surfaces (Bux *et al.*, 1994). The quantity of bacterial EPS in activated sludge is controlled by the concentrations of various nutrients in the growth medium, sludge retention time and oxidation of polymers by other bacterial species present (Brown and Lester, 1979).

Sutherland (1985) reviewed the composition of bacterial EPS and suggested that EPS produced by certain bacteria is almost entirely composed of neutral sugars and a limited number of uronic acids. Uhlinger and White (1983) highlighted the fact that uronic acids

are a unique anionic component of polysaccharides found external to the cellular cytoplasmic membranes. Some polysaccharides contain amino sugars or sugars with amine or amide-linked functional groups. Little is known about the nature and differences in monomer composition between domestic and industrial sludge polysaccharides. Horan and Eccles (1986) isolated an EPS fraction from sludge which contained glucose, galactose, mannose, glucuronic acid and galacturonic acid.

Structural investigations of isolated polysaccharides must be undertaken after careful purification and establishment of the homogeneity of the product. This is an important step in the chemical characterisation of the EPS and the determination of the relationship between chemical structure and biological function. Because of the variations in the types of residues and linkages between residues there are an enormous number of polysaccharides in nature. Several criteria for purity can be used to assess the purity of polysaccharide preparations.

These are:

- (a) Constancy in monosaccharide composition,
- (b) uniform behaviour on gel filtration or ion-exchange chromatography, and
- (c) Constancy in quantitative values.

4.1.1 Importance of extracellular polysaccharides characterisation

It has been demonstrated that purified EPS from bacterial cultures may take up and concentrate metal ions from solution (Brown and Lester, 1980). Extracellular polysaccharides have also been frequently cited as an important factor that determines the heavy metal uptake capacity of activated sludge (Forster, 1980; Brown and Lester, 1979; Swalaha, 1993), yet only a limited characterisation of the metal binding properties of extracted material exist. The amount of polysaccharides required for optimal aggregation during flocculation is related to the total surface area, molecular weight and monomer composition (Harris and Mitchell, 1973).

It is apparent that the nature, composition and molecular weight of sludge exocellular polysaccharides are of fundamental importance in determining the settling properties of activated sludge. For the elucidation of the complete molecular structure the structural features that need to be determined are, for example, the types of monosaccharide residues present, the number of residues per molecule and the position of glycosidic linkages between residues. The common analytical techniques used in the structural analysis of polysaccharides include periodate oxidation, paper chromatography and gel filtration chromatography.

Therefore the objectives of this part of the study were to apply chromatographic techniques for the separation of different polysaccharide fractions which constitute the activated sludge polysaccharides, and to determine their chemical and monomer composition.

4.2 HYPOTHESIS

Activated sludge extracellular polysaccharides are high molecular weight compounds which can be separated into different fractions to aid in their characterisation.

4.3 AIMS

- (a) To apply chromatographic techniques in the separation and purification of different polysaccharide fractions which constitute activated sludge polysaccharides.
- (b) To characterise extracellular polysaccharides.

4.4 OBJECTIVES

- (a) To separate extracellular polysaccharide fractions using ion-exchange chromatography.
- (b) To separate EPS fractions by gel filtration.
- (c) To analyse the structure of EPS fractions by periodate oxidation.
- (d) Determination of monomers by paper chromatography.
- (e) To determine chemical composition of the EPS.

4.5 MATERIALS AND METHODS

4.5.1 Ion exchange chromatography

Purified polysaccharides obtained after extraction and purification procedure (Chapter 3) were dissolved in 100 mM Tris/HCl buffer (pH 9) and applied to a column (1.5 cm by 45 cm) of DEAE-Sepharose CL-6B equilibrated with the same buffer. Fractionation using a stepwise pH gradient from pH 9 to pH 4 at a flow rate of 10 ml per hour. Fractions of 10 ml were collected and the elution was spectrophotometrically monitored by continuously measuring the UV absorbance at 260 nm and 280 nm. This was done to indicate the presence of nucleic acids and proteins. Total sugar content in each fraction was determined by phenol/sulphuric acid assay (Dubois *et al.*, 1958). Uronic acids were measured by the carbazole/sulphuric acid method with D-glucuronic acid as the standard (Bitter and Muir, 1962).

4.5.2 Gel filtration

Determination of molecular weight distribution in purified EPS samples was conducted with Sepharose CL-6B. Small freeze-dried samples were chromatographed on a column of Sepharose CL-4B equilibrated and eluted with 100 mM Na_2HPO_4 - NaH_2PO_4 (pH 7) containing 0.5 M NaCl. Flow rate was kept at 10 ml/h.

Elution was monitored continuously measuring of UV absorbance at 260 nm or 280 nm. Analysis of fractions were carried out as above.

4.5.3 Acid hydrolysis before paper chromatography

Dried polysaccharide (10 mg) was hydrolysed at 100 °C for 5 to 24 h in sealed glass vials with 15 ml of 2 M trifluoroacetic acid (TFA). After hydrolysis unreacted TFA was removed by evaporation under reduced pressure. Trifluoroacetic acid residues were removed from sugar monomers with ammonia in aqueous methanol .

Complete hydrolysis of uronic acids was performed by suspending freeze-dried polysaccharide (10 mg) in 0.2 ml of ice-cold 80% (v/v) H₂SO₄. The suspension was left overnight in a 2 °C freezer. Distilled water (2.8 ml) was added, and the solution was heated at 100 °C for 5 hours.

4.5.4 Paper chromatography

Ascending paper chromatography (Whatman Grade 20 Chr) was performed with the following solvents:

- (a) 1-butanol:pyridine:HCl(0,1 M) (5:3:2, vol/vol)
- (b) 2-propanol:acetic acid:water (58:8:18,vol/vol)
- (c) pyridine:ethyl acetate:acetic acid:water (5:5:1:3, vol/vol)
- (d) benzene:1-butanol:pyridine:water (1:5:3:3, vol/vol)

(e) 1-butanol:pyridine:water (6:4:3, vol/vol)

Neutral sugars were chromatographed in solvents D and C, acid-labile sugars in solvents A and B, uronic acids in solvent E and amino sugars chromatographed in solvent E. This method was developed by Christensen *et al.*, (1985)

4.5.5 Periodate oxidation

Fractions which showed highest sugar concentration during gel filtration were pooled and oxidized by 0.1 M sodium periodate to determine the number of sugar residues present in each polysaccharide (Ikeda *et al.*, 1982). Oxidation by periodate is one of the methods which was used for end-group assay. A linear polysaccharide yields formic acid from the non-reducing terminal unit and formic acid and formaldehyde from the reducing end unit. Excess oxidant was removed by adding ethylene glycol and the formic acid titrated directly by sodium hydroxide (*Appendix E*).

4.5.6 Amino acid analysis

Purified extracellular polysaccharide was analysed for amino acid content using a Beckman Amino Acid Analyser.

4.6 RESULTS

4.6.1 Ion-exchange chromatography

The presence of several different polysaccharide fractions was demonstrated by anion-exchange chromatography. The elution profile of the EPS is shown in Figs. 4.1 and 4.2. The behaviour of the polysaccharides on ion-exchange resins yielded information about the purity of a polysaccharide preparation. Domestic polysaccharide was separated into eight fractions that formed three different groups which were identified at acidic, neutral and basic pH ranges. These were pooled and identified as Fraction A, Fraction B and Fraction C respectively. By decreasing the pH from pH 9 to pH4 the first polysaccharide (Fraction A) was eluted at pH 8.5. The second polysaccharide (Fraction B) was eluted at a slightly acidic pH (less than pH 7) while the third polysaccharide (Fraction C) was eluted at pH 4. The majority of polysaccharide fractions were eluted at a pH which was less than 7. This indicates the net negativity of most of the EPS components eluted.

Polysaccharide eluted from industrial sludge had a distorted elution profile with a sharply rising front and considerable tailing while domestic sludge polysaccharide showed a sharply rising front only. There were approximately sixteen separated fractions in the industrial polysaccharides preparation and the

majority were in the neutral pH range. Overall, the components from both industrial and domestic sludges were well separated into acidic, basic and neutral groups.

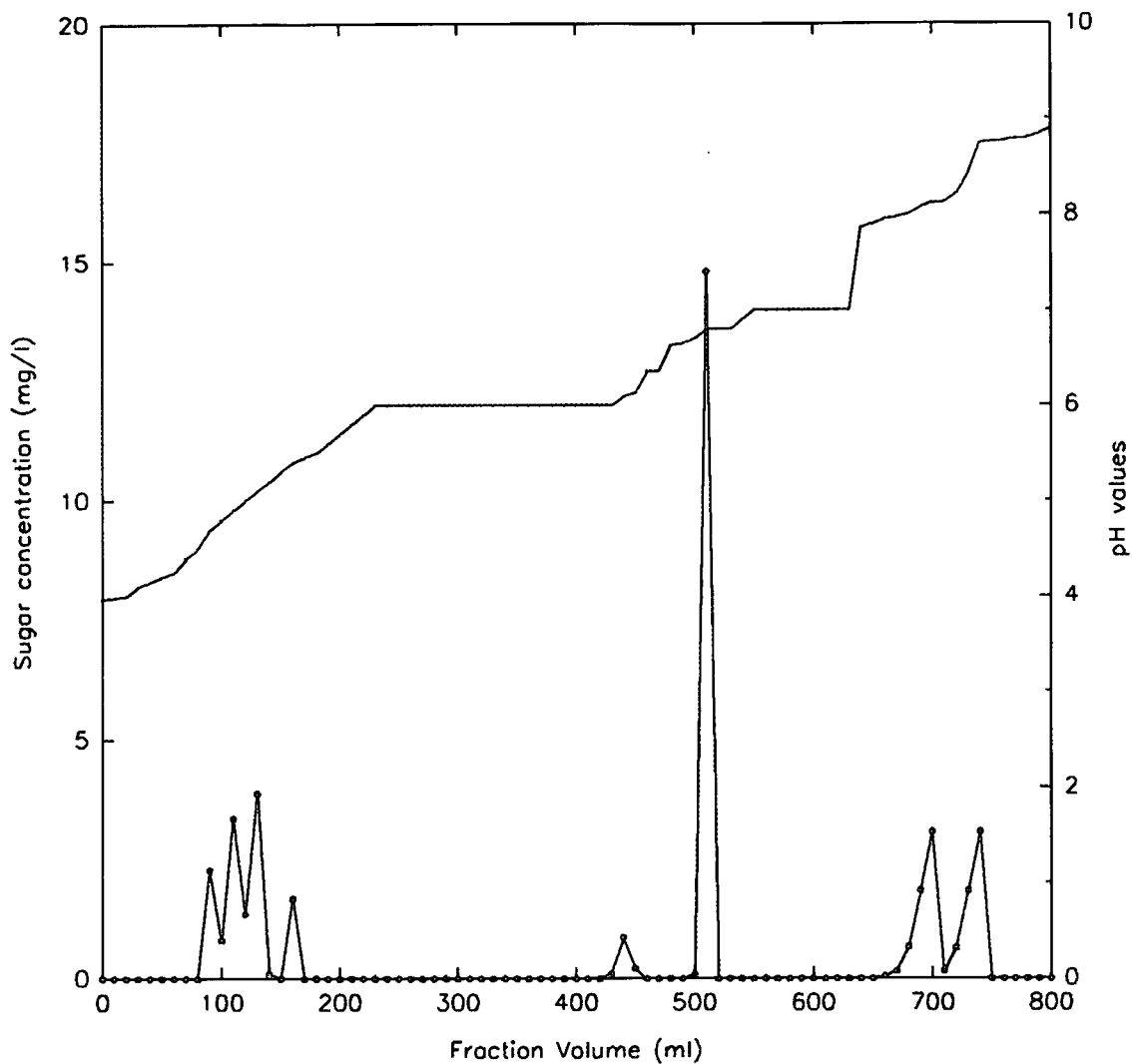


FIGURE 4.1. Ion-exchange chromatography of the purified polysaccharide fraction, extracted from a domestic sludge, on a column (1.5 cm by 45 cm) of DEAE-Sepharose CL-6B. The eluent was 100 mM Tris/HCl buffer with a pH gradient and a flow rate of 10 ml/h. Fractions (10 ml) were analysed for total sugars.

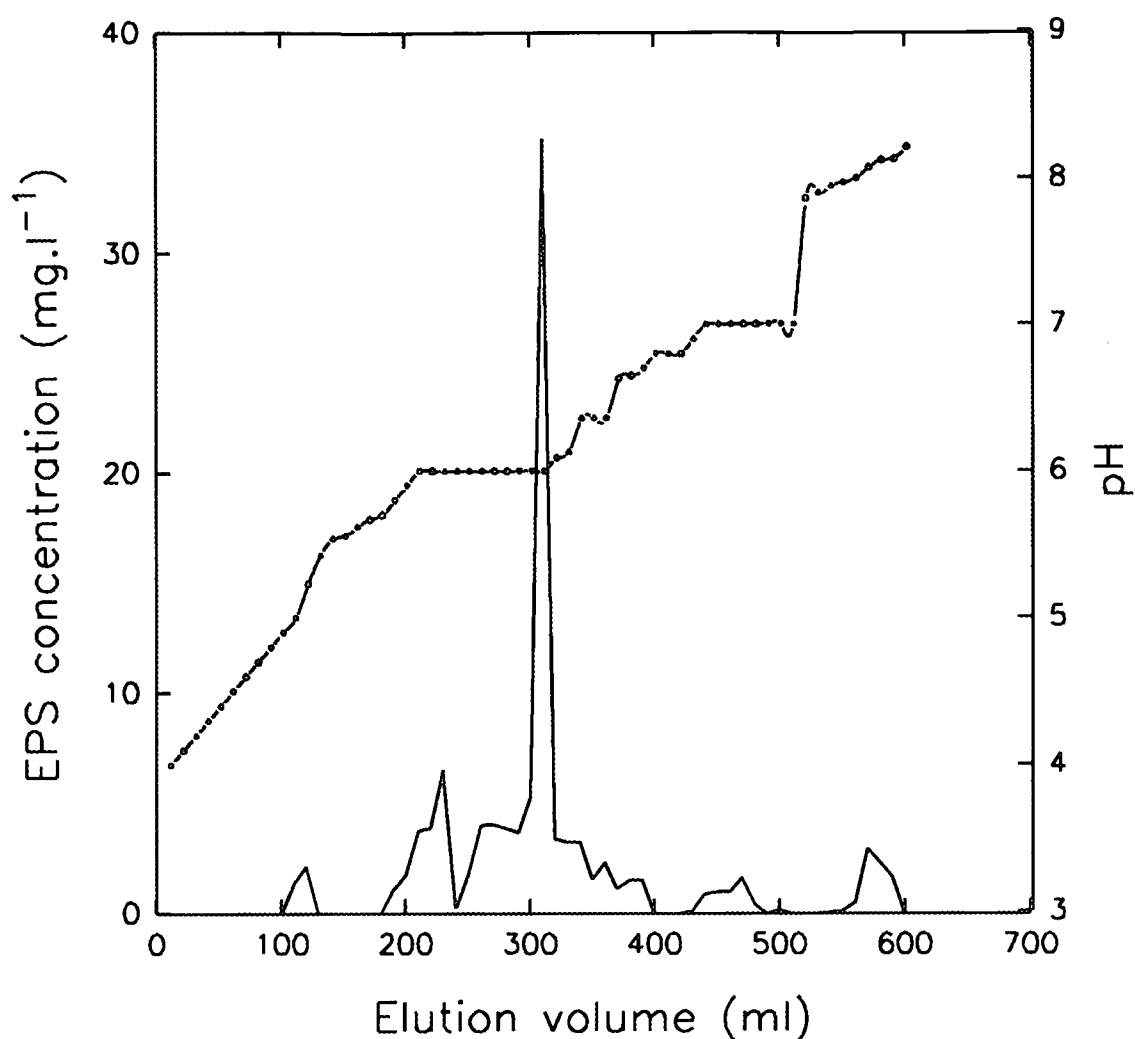


FIGURE 4.2. Ion-exchange chromatography of the purified polysaccharide fraction extracted from an industrial sludge on a column (1.5 cm by 45 cm) of DEAE-Sepharose CL-6B. The eluent was 100 mM Tris/HCl buffer with a pH gradient and a flow rate of 10ml/h. Fractions (10 ml) were analysed for total sugars.

4.6.2 Gel filtration chromatography

Gel filtration results showed a wide distribution range of sludge extracellular polysaccharide from both domestic and industrial EPS. In industrial polysaccharide a widely distributed peak with two or more minor fractions were eluted which indicates a distribution of molecular weights (Fig. 4.3), while in the domestic sample a minor peak was eluted first followed by a widely distributed peak (Fig. 4.4). These separated fractions were pooled and the number of monomer residues estimated by the periodate oxidation method.

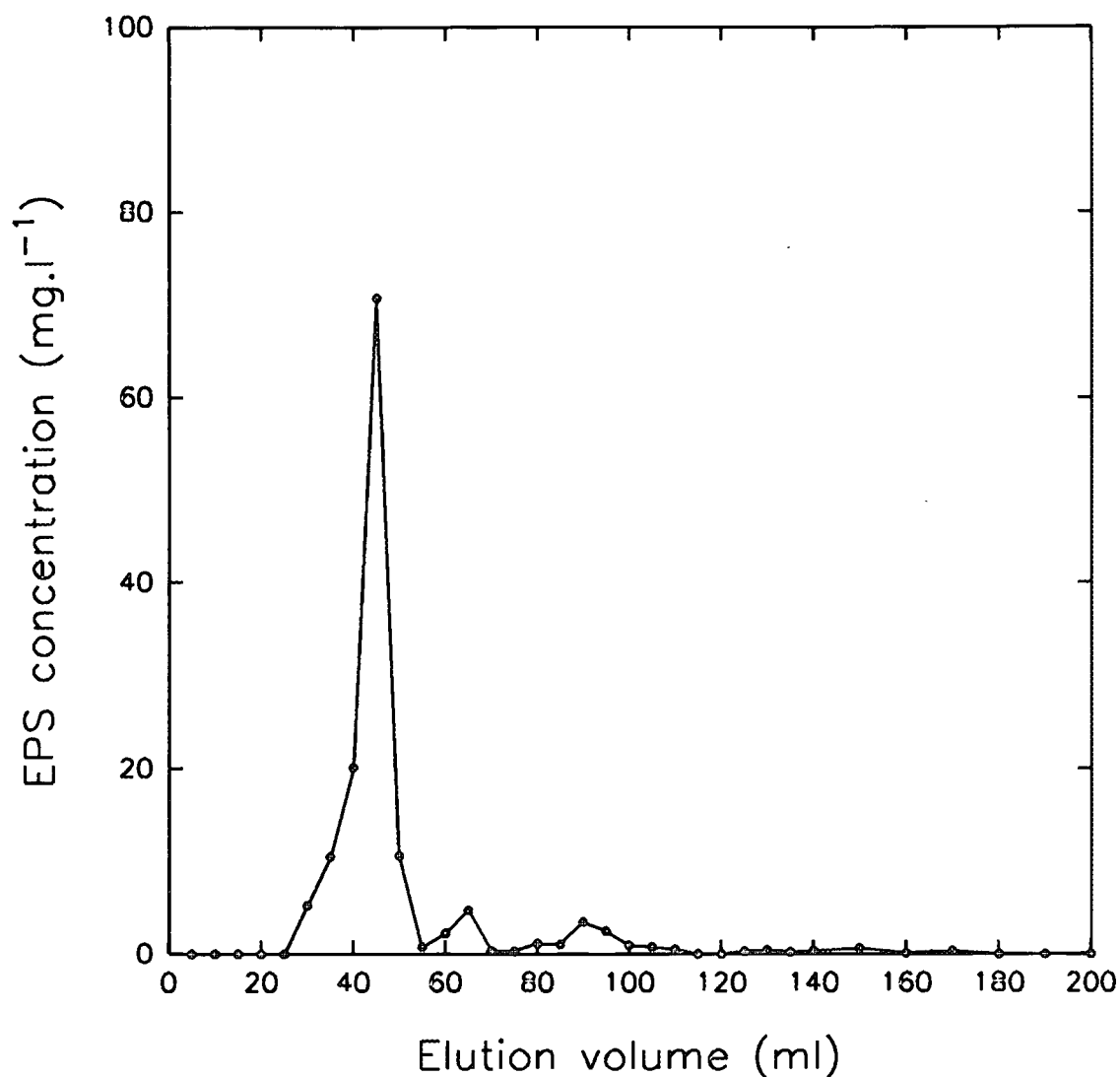


FIGURE 4.3. Gel filtration of the purified polysaccharide fraction from industrial sludge on a column (1.5 cm by 50 cm) of Sepharose CL-4B. The eluent was 100 mM sodium phosphate (pH 7) containing 0.5 M NaCl. Flow rate was 10 ml/h. Fractions of 5 ml were collected and analysed for carbohydrate content.

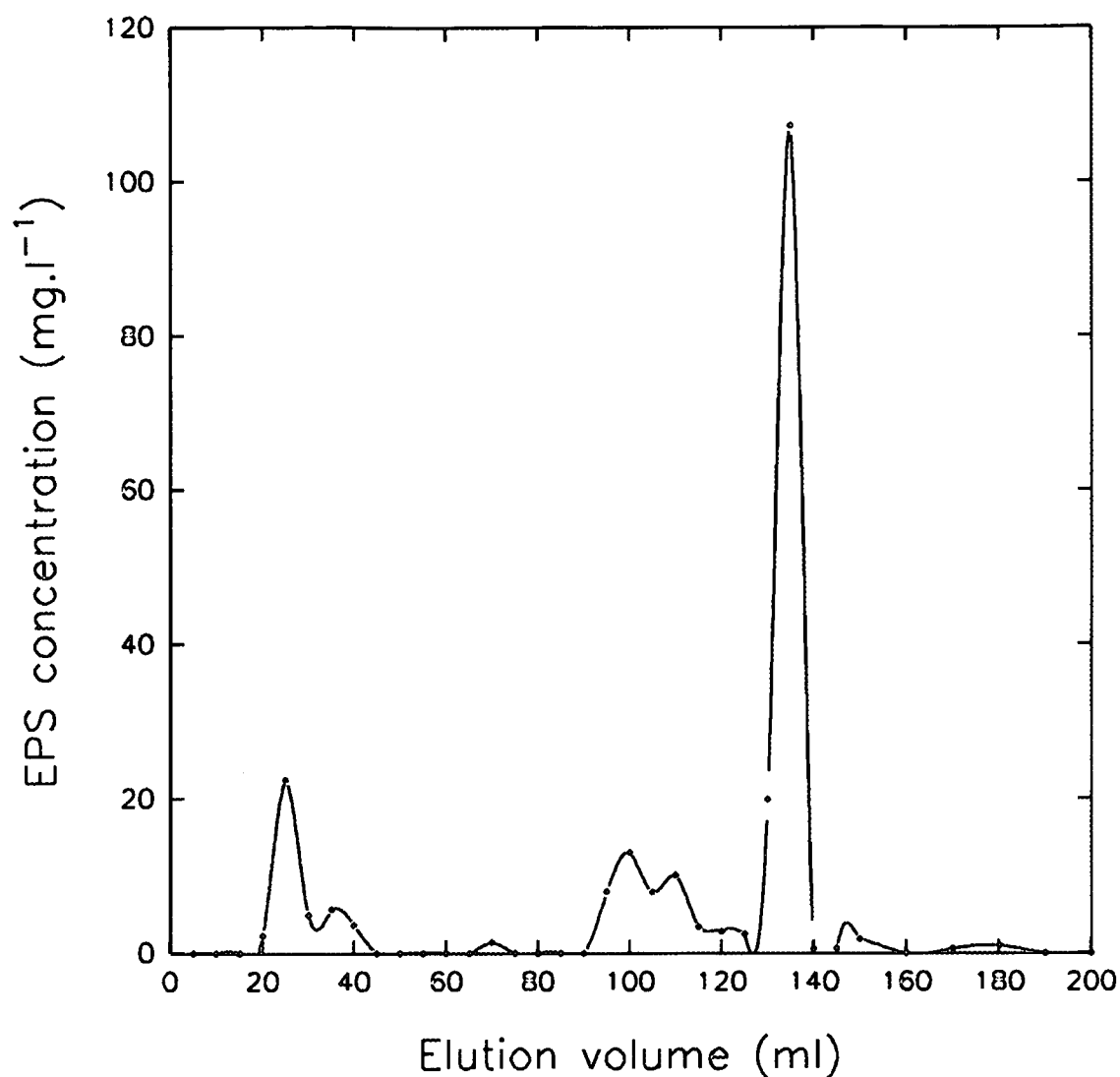


FIGURE 4.4. Gel filtration of the purified polysaccharide fraction from domestic sludge on a column (1.5 cm by 50 cm) of Sepharose CL-4B. The eluent was 100 mM sodium phosphate (pH 7) containing 0.5 M NaCl. Flow rate was 10 ml/h. Fractions of 5 ml were collected and analysed for carbohydrate content.

4.6.3 Paper Chromatography

In determining monosaccharides present in purified extracellular polysaccharide samples R_f values for a mixture of sugars typically encountered in extracellular polysaccharides of bacteria found in activated sludge were determined. Paper chromatography after acid hydrolysis showed three components which co-chromatographed with glucose and fructose (stained with *p*-anisidine hydrochloride) (Table 4.1 and 4.2), galactose amine (stained with ninhydrin) (Table 4.3 and 4.4) and glucuronic acid (stained with *p*-anisidine HCl) (Table 4.5). Two other neutral monosaccharides from both types of polysaccharides were observed but could not be identified with any of the standards employed.

TABLE 4.1. Determination of R_f values of monomers present in industrial sludge EPS chromatographed in solvent C (neutral sugar monomers).

Monosaccharides	R_f values
Fructose	0.614
Mannose	0.628
Galactose	0.537
Glucose	0.575
Monomer-1	0.820
Monomer-2	0.575
Monomer-3	0.380

TABLE 4.2. Determination of R_f values of monomers present in domestic sludge EPS chromatographed in solvent C (neutral sugar monomers).

Monosaccharides	R_f values
Fructose	0.614
Mannose	0.628
Galactose	0.537
Glucose	0.575
Monomer-1	0.856
Monomer-2	0.575
Monomer-3	0.614

TABLE 4.3. Determination of R_f values of monomers in industrial EPS chromatographed in solvent D (amino sugar monomers).

Monosaccharides	R_f values
Glucosamine	0.363
Galactosamine	0.345
Monomer-1	0.726
Monomer-2	0.262
Monomer-3	0.345

TABLE 4.4. Determination of R_f values of monomers in domestic EPS chromatographed in solvent D (amino sugar monomers).

Monosaccharides	R_f values
Glucosamine	0.363
Galactosamine	0.345
Monomer-1	0.476

TABLE 4.5 Determination of R_f values of monomers in domestic and industrial EPS chromatographed in solvent E (hexuronic acid monomers).

Monosaccharides	R_f values
Glucuronic acid	0.877
Galacturonic acid	0.346
Monomer-1	0.876

In Tables 4.1, 4.2, 4.3, 4.4 and 4.5 are R_f values of unknown components and known sugars chromatographed in different solvents. In Table 4.1 three unknown spots were observed. Unknown monomer-3

migration was similar to fructose migration (Table 4.2). The migration of both galactose and glucose was too close to each other such that their identification with unknown monomer-2 using paper chromatography was unsuccessful. This unknown monomer was eventually identified with glucose after repeated application of paper chromatography. Unknown monomer-1 could not be identified with any of the neutral sugars, but it was later identified as glucuronic acid (Table 4.5).

One other unknown monomer (monomer-3) from industrial polysaccharide was identified in Table 4.3 with ninhydrin spray and had similar R_f value with that of galactosamine. The other unknown monomers in the same group of amino sugars could not be identified with either glucosamine or galactosamine. The observed spot in the domestic polysaccharide fraction could also not be identified with either of these amino sugars (Table 4.4).

The chromatographic analysis of industrial and domestic EPS migrated in solvent E showed one type of monomer component with migration pattern which was similar to glucuronic acid (Table 4.5). It is therefore clear, that the industrial EPS has glucose, galactosamine, glucuronic acid and three other neutral/amino monosaccharides which could not be identified with any of the monosaccharides used in the present study. The domestic polysaccharides showed glucose, fructose, glucuronic acid and one unidentified amino sugar.

4.6.4 Periodate oxidation

TABLE 4.6. Periodate oxidation of chromatographed extracellular polysaccharide fractions from activated sludge.

Fraction	NaIO ₄	HCOOH
	consumed (mmoles)	produced (mmoles)
A-Industrial	0.254	0.131
B-Domestic	0.140	0.0467
C-Domestic	0.220	0.8675

Results of complete periodate oxidation of the separated fractions are summarized in Table 4.6. Considerable differences in the number of residues calculated for each fraction was observed. One polysaccharide fraction from industrial sludge and two fractions from domestic polysaccharides separated by gel filtration were investigated by the periodate oxidation process.

Because of the lack of known reference sugars which could be used it was difficult to calculate the actual number of residues present in each fraction. However, the basic assumption made was that fraction C had the highest number of residues and fraction A had the lowest. This was calculated from the sodium periodate consumed

after hydrolysis. The above results compare reasonably with those supported by Ikeda *et al.*, (1982) on the EPS of *Zoogloea ramigera* 115 which is usually found in activated sludge biomass.

4.6.5 Amino acid analysis

Table 4.7 Amino acid composition of the extracted Industrial and Domestic sludge EPS. (The results were calculated as % of amino acids in 1 ml of water (1 ml of water=1.4962 gram)).

Amino acid	Domestic EPS	Industrial EPS
Glutamine	0.020955	0.004182
Serine	0.003827	0.001443
Histidine	0.002366	0.000624
Asparagine	0.018662	0.003676
Threonine	0.006248	0.001812
Proline	0.006651	0.01634
Glycine	0.008430	0.003384
Alanine	0.010827	0.002470
Valine	0.010829	0.002536
Methionine	0.002617	0.000603
Isoleucine	0.007670	0.001417
Leucine	0.012672	0.002635
Tyrosine	0.004903	0.00710
Phenylalanine	0.007844	0.001632
Lysine	0.005656	0.001498
Argenine	0.005327	0.001494

At the end of the purification procedure the quality of EPS was found to be higher in industrial sludge than in domestic sludge (Table 3.1 and Table 3.2). This was also confirmed by comparing the results obtained in the amino acid analysis of samples from both sludges (Table 4.7) where domestic sample was found to contain a larger percentage of amino acids than industrial sludge samples. Amino acids determined in both samples were; glutamine, serine, histidine, asparagine, threonine, proline, glycine, alanine, valine, methionine, isoleucine, leucine, tyrosine, phenylalanine, lysine and arginine. These differences in EPS/protein content may not be a significant factor since Brown and Lester (1979) suggested that the quantity of protein and carbohydrates is different for different sludge samples.

4.7 DISCUSSION

Extracellular polysaccharides play an essential role in biofilm structure, activity and performance in biological wastewater treatment. They mediate the transport of chemicals to and from microorganisms (Zhang *et al.*, 1997). They also have ion-exchange properties due to negatively charged functional groups (Sutherland, 1977).

Naturally occurring polysaccharides vary greatly in molecular size ranging from several thousands to millions of daltons (Sutherland, 1977). The molecular structure and architecture is diverse but can be divided into three major types, a linear structure in which the units are joined by the same type of glycosidic linkage to form long linear chains, a substituted linear structure in which monosaccharides or short side chains of oligosaccharide units are attached to the main chain, and a branched structure in which long side chains are attached to the main chain and to side chains. Some polysaccharides contain glycoproteins which result from the covalent association of carbohydrate moieties with proteins (Ford *et al.*, 1991). Glycoproteins are widely distributed in animals, plants and microorganisms.

Large amounts of EPS eluted by ion exchange chromatography were shown to have either a neutral or negative charge (Fig. 4.1 and

4.2). Several hypotheses have been postulated to explain this complex phenomenon (Forster, 1976; Horan and Eccles, 1986). Stumm and Morgan (1962) found the principal ionogenic component of sludge polysaccharide at neutral pH to be glucuronic acid, which contributes a strong negative charge. Therefore a conclusion can be made that negative charge by an EPS eluted near pH 7 is contributed by glucuronic acids.

The ion-exchange results present some interesting questions. In particular, are the fractions that have been detected merely fragments of a single main polysaccharide, precursors of the main polysaccharide, or separate ? These questions arise from the inconsistencies of the ion-exchange and gel filtration results.

The gel filtration results indicate that either different polysaccharides fractions which were revealed by ion-exchange chromatography have molecular weight distribution which is in a same range or the technique used produced poor resolution (Fig. 4.3 and 4.4). These results confirmed what Forster (1985) observed on sludge extracellular polymer samples. His work demonstrated a wide range of molecular weights using gel filtration. He observed the average value to be one million.

These results also highlight the very real problem in monitoring the eluted chromatographic EPS fractions using Dubois *et al.*, (1958) method. This is because of the lack of a method which can

simultaneously detect different types of polysaccharides. This shortcoming may cause erroneous assessment of the fractions.

The application of paper chromatography facilitated identification of a mixture of all those sugar monomers thought likely to be encountered in activated sludge extracellular polysaccharides. The monomer composition of domestic and industrial sludge polysaccharides showed a remarkable similarity, with only four monomers being detected. The unidentified monomers are thought to be those monomers which were not used as standards in the present study. Some of these monomers may be covalently bonded to aliphatic, aromatic, sulphate and amino groups which are thought to be found in the activated sludge EPS (Horan and Eccles, 1986).

Friedman *et al.*, (1968) observed the structure of polysaccharide, extracted from *Z. ramigera* 115 which is usually found in activated sludge, to be a branch network of fibrous strands. Ikeda *et al.*, (1982) showed that the zoogloeal material purified from *Z. ramigera* 115 is a branched polysaccharide having a possible repeating unit structure. They concluded that this structure was a polysaccharide similar to cellulose. Other researchers have found extracellular polymers in different species which had a structure similar to cellulose (Dudman and Wilkinson, 1956).

The present results indicated that domestic polysaccharides have more residues than industrial fractions. However, the periodate

oxidation results (Table 4.6) did not give enough information such that the sequence of the core chain, length, degree of branching and sequence of each branching residue could be determined. Elucidation of these aspects is certainly required. It is clear that the determination of monosaccharide composition of EPS gives an understanding on the number of EPS variations found in sludge EPS.

During the present study uronic acid content was found to be greater in domestic sludge than in industrial sludge samples. But the uronic acid determination, by the method of Bitter and Muir (1962), proved to be difficult since the effect of contaminants distorted the results. Bitter and Muir (1962) noted that the effect of glucose contamination in the sample could not be avoided. Even though interference by other carbohydrates could be avoided, glucose yielded 13% of the colour of glucuronic acid. They suggested that this method should be used only in polysaccharides containing no glucose residues or the correction factor should be used to eliminate glucose effect. The findings of the present study, therefore, concur with those of Bitter and Muir (1962).

Throughout this investigation results of both industrial and domestic sludge polysaccharides have indicated the presence of EPS having different charge characteristics. It is also clear that the major components were either negatively charged or neutral. Periodate oxidation indicated that polysaccharides are composed of

long-chain species. These polysaccharide chains consists of groups which are either negative or positively charged.

It may be inferred, therefore that polysaccharide material excreted or exposed at the surface of micro organisms found in activated sludge contains functional groups that are primarily anionic and nonionic in most neutral pH ranges. These results contradict those of Pavoni *et al.*, (1972) whose observations from pure cultures that at pH values between 3.0 and 7.0 exocellular polymers do not flocculate bacteria. They postulated that between pH 3.0 and pH 7.0 there may not be active bonding sites on either the bacterial or polymer surface. Their findings may be attributed to the denaturing effect of the bacterial polymer caused by the ethanol extraction procedure.

4.8 CONCLUSIONS

Extracted extracellular polysaccharide appeared to contain functional surface groups that are primarily anionic and nonionic in most pH ranges. The chromatographic results demonstrate the potential for variety in the polysaccharide content of the activated sludge. The results indicate that the activated sludge samples used contained a mixture of polysaccharides.

The extraction products from pure cultures have been shown to consist of hexoses, hexuronic acids and hexosamines, which have been confirmed by the results of the present study. Therefore it seems likely that the proteins associated with the present sludge EPS are part of the polysaccharide structure or are products of either cellular disintegration or autolysis. In living cells, polysaccharides may be combined covalently with members of other classes of compounds notably proteins and lipids (Goodwin and Forster, 1985).

Such a mixture is not unlikely in view of the variety of microbial species, producing different polysaccharides, which have been reported to be present in activated sludge (Wallen and Davis, 1972).

This observation may expand our understanding on the mechanism of bioflocculation with regard to electrostatic bonding. Therefore the objective of the next phase of the project was to investigate the capability of EPS to biosorb metals individually and in combination.

CHAPTER FIVE

5.0 DETERMINATION OF EXTRACELLULAR POLYSACCHARIDE METAL BIOSOPTIVE CAPABILITY

5.1 INTRODUCTION

The ability of microorganisms to accumulate heavy metals is a well documented phenomenon (Kasan, 1993). This method is regarded as a powerful tool against heavy metal toxicity (Wnorowski, 1991). The microbial population most predominant in sludges are the polymer-producing eubacteria (Swalaha, 1993).

The uptake of heavy metals from solutions by microorganisms is called biosorption and several mechanisms may be involved, including adsorption, co-precipitation, ion-exchange, complexation as well as processes dependent on metabolism (Tsezos, 1985). Bux *et al.*, (1996) have demonstrated the potential of activated sludge to biosorb metals from solution. Activated sludge could prove an inexpensive biosorbent because it is produced in large amounts by sewage treatment plants, and is more readily available than single microbial species. Cell walls of microbial biomass (found in activated sludge), mainly composed of polysaccharides, proteins,

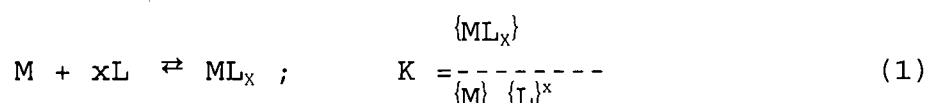
and lipids, offer particularly abundant metal-binding functional groups such as carboxylate, hydroxyl, sulphate, phosphate and amino groups (Forster, 1976). Furthermore, the physico-chemical phenomenon of metal biosorption, based on adsorption, ion-exchange, complexation and microprecipitation, is relatively rapid and can be reversible (Tsezos and Volesky, 1981; Kuyucak, 1987; Kuyucak and Volesky, 1988; Volesky, 1994)

Solution pH affects the solution chemistry of the metals, the activity of functional groups in the biomass as well as the competition of metallic ions for the binding site (Pavoni *et al.*, 1972). At pH 4-5, almost all metallic species, copper, zinc, and cadmium are ionized as various cationic species. At that pH the carboxylate groups of the biomass will largely be dissociated generating negatively charged surfaces. Electrostatic interactions between cationic species and negatively charged cell surfaces may be responsible for the metal binding.

5.1.1 Adsorption theory

Metal binding can be characterized in terms of the number of binding sites available, the number and types of site and the ability of the "molecule" to form complexes. This final characteristic, which may also be thought of as a measure of the strength of the binding, is determined as the conditional stability constant (K) for each of the metal/polymer systems (Rudd *et al.*, 1983).

The equilibrium reaction for complex formation can be expressed as:



Conditional complexation parameters could be experimentally derived by expressing the bound metal concentration $\{ML_x\}$ as free metal $\{M\}$ subtracted from the total metal present, and $\{L\}$ as the concentration of available binding sites, effectively the complexation capacity.

The relationship between concentrations of bound and free metal over a range of added metal concentrations has been described by Ruzic (1982):

$$\frac{ML_x}{L^x} = \frac{M}{L} + \frac{1}{LK'} \quad (2)$$

This straight line relationship could be solved graphically where M/ML_x is plotted against $\{M\}$, which produces a line with slope $1/L$ and intercept $1/LK'$, where K' is the conditional stability constant. Deviations from a straight line relationship occur when more than one complex is formed, or when the stoichiometry of the complex is not 1:1. Such deviations can be characterized by extensions to equation 2 developed by Ruzic (1982).

Evaluation of the mechanism of biosorption can also be ascertained by applying the Langmuir Adsorption Model. Extensive research on the equilibrium of biosorption of metals has shown that biosorption systems follow an adsorption-type isotherm (Kuyucak, 1987).

The extent of biosorption of a metal ion by microbial biomass from a single-solute solution is a function of the equilibrium metal-ion concentration in solution, all other parameters such as pH and temperature remaining constant. Brunauer *et al.*, (1940) (cited by Ruthven, 1984) have classified the isotherms for physical adsorption into five classes.

The isotherms for the true microporous adsorbents in which the pore size is not very much larger than the molecular diameter of the sorbate molecule, are normally of type 1. This is because for such adsorbents there is a definite saturation limit corresponding to complete filling of the micropores.

Occasionally if intermolecular attraction effects are large, an isotherm of type V is observed. An adsorption isotherm of type IV suggests the formation of two surface layers either on a plane surface or on the wall of a pore very much wider than the molecular diameter of the sorbate. Isotherms of types II and III

are observed in adsorbents containing a wide range of pore sizes.

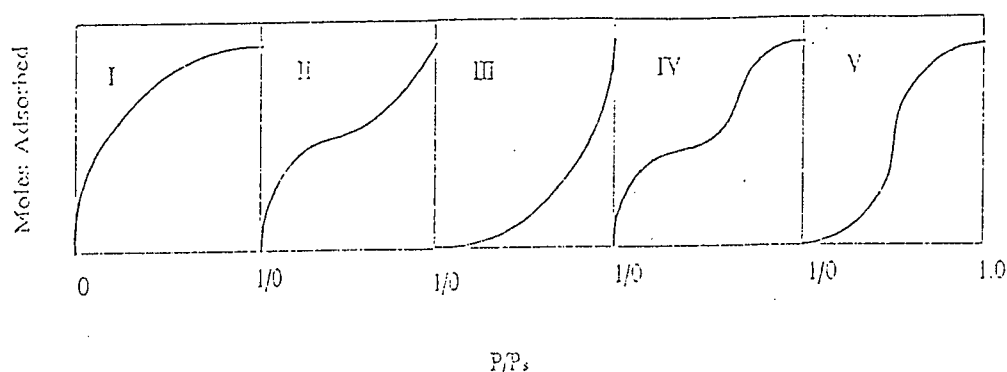


Fig. 5.1 Brunauer classification of adsorption isotherms (Hughes and Poole, 1989).

In each system there is a continuous progression with increasing loading from monolayer to multilayer adsorption and then to capillary condensation (Gasser, 1985). The preferred form for depicting this distribution is to express the quantity bound q_s as a function of C , at fixed temperature, the quantity q_s being the amount of solute adsorbed per unit weight of adsorbent and C the concentration of solute remaining in solution at equilibrium.

The concepts of Langmuir (1981) and Nelson *et al.*, (1981) centre on the use of metal adsorption plots which permit the analysis of such data to predict the nature and efficiency of metal adsorption to activated sludge. If q_e , the amount of metal adsorbed to sludge is plotted against C as the concentration of free metal at equilibrium, S- or L-shaped isotherms are obtained. The S-curve is indicative of competitive adsorption with moderate intermolecular attraction between the adsorbed metal species. The L-curve indicates a reduction of available binding sites as C increases, causing the proportion of total metal bound, q_e , at higher concentration to decrease.

A derivation of the Langmuir model allows for linearizing of data and shows how far the sorption process is in agreement with the model:

$$1/q_e = 1/Q^\circ + (1/bQ^\circ) (1/C) \dots\dots\dots (1)$$

where b is the constant related to the energy or net enthalpy of adsorption, Q° is the number of moles of solute adsorbed per unit weight of adsorbent in forming a complete monolayer on the surface, q_e and C are as defined previously (Volesky, 1987).

The data from either S- or L-shaped isotherms may be converted by plotting $1/q_e$ against $1/C$. If the linear plots result then the Langmuir Model of Adsorption (Langmuir 1981) may be used to

interpret data to determine equilibrium constants which describe sludge/metal interactions. $K_a = bQ$ is a measure of the overall affinity (i.e. a combination of the number of binding sites available and the binding intensity) whilst $X_m = Q^\circ$ measures the binding capacity of the polysaccharide surface. b quantifies the relative strength of the binding interactions whereas K_a depends of the position of the equilibrium between the metal ions and the receptor ligand. As such the relative position of the metals in the series may depend on the number of sites available to a metal as well as the binding capacity (Forster, 1985).

Another model which can be used to interpret metal adsorption data is the Freundlich adsorption model. The Freundlich adsorption model does not become linear at low concentration but remains concave to the concentration axis; and it does not show a saturation or limiting value (Volesky, 1987). The general form of this model is

$$q_e = kC^{1/n}$$

This can be linearized by taking logarithm of both sides of the equation to give:

$$\log q_e = \log k + 1/n \log C$$

The intercept $\log k$ gives a measure of the adsorbent capacity and

the slope $1/n$ gives the intensity of adsorption (Adamson, 1976).

5.1.2 Metal biosorption by extracellular polysaccharides

Many polymers of biological origin are known to bind metals, e.g. microbial exopolymers (Mittelman and Geesey, 1985). The capability of biomaterials, especially biopolymers derived from microorganisms (Friedman and Dugan, 1986) and plants, to chelate heavy metals has been applied to the recovery of heavy metals from mine drainages and industrial wastewaters (Patterson, 1987)

There has been considerable interest in the role of bacterial extracellular polysaccharides in metal binding by activated flocs. Dugan and Pickrum (1972) proposed that capsular EPS plays an important role in the adsorption of heavy metals by wastewater bacteria. They have shown that eight species of floc-forming bacteria isolated from wastewaters adsorbed metal ions. The amount of metal uptake varied with the isolate.

Bacterial EPS from cultures of *K. aerogenes* and activated sludge simulations showed biosorptive capability for cadmium, nickel and cobalt. Other workers have observed complexation of metals with polymers extracted from pure bacterial cultures and from

activated sludge (Forster and Lewin, 1972). Swalaha (1993) reported that the adsorption of zinc by six EPS-producing organisms did not increase proportionally to the amounts in solution. This indicated a fixed number of sites for adsorption.

The importance of polymer-metal interactions and the mechanisms of polymer production, binding of soluble and insoluble metals and precipitation have been reviewed by Brown and Lester (1979).

Metal biosorption by EPS from activated sludge have been investigated by Foster (1983) while Brown and Lester (1982) investigated the effects of metal concentration on metal biosorption. During these investigations extracellular polymers were of a very low quality because extraction techniques were harsh and purification techniques were not applied.

5.1.3 Mechanisms of biosorption

One of the primary driving forces for adsorption results from a specific affinity of a solute for a solid. Three principle types of adsorption are distinguishable:

1. Electrical attraction of the solute to the adsorbent
2. Van der Waals attraction
3. A chemical nature (Volesky, 1987)

Adsorption of the first type falls within the scope of ion-exchange and is often referred to as exchange adsorption. Exchange adsorption is a process in which ions of one substance concentrate at a surface as a result of electrostatic attraction to charged sites at the surface. For two potential ionic adsorbates in like concentrations and in the absence of the specific sorption effects, the charge on the ion is the determining factor for exchange adsorption (Volesky, 1990). In the system with a monovalent ion and a trivalent ion under the stated conditions the influence of kinetic energy to remain in the solution phase is the same for each, but the electronegativity of the ion causes it to be attracted more strongly toward a site of opposite charge on the surface of the adsorbent. For ions of equal charge, molecular size determines the order of preference of adsorption, the smaller ion being able to accomplish a closer approach to the adsorption site and thus being favourable (Volesky, 1990).

These chemical adsorption processes exhibit high energies of adsorption because the adsorbate forms strongly localized bonds of active centres on the adsorbent. Chemical interactions between the adsorbent and the adsorbate is favoured by higher temperatures since chemical reactions proceed more rapidly at elevated temperatures than at low temperatures (Volesky, 1990).

5.1.4 Metal biosorption in a binary mixture

The degree of removal of heavy metals from wastewater by biosorption depends on the multimetal competitive interactions in solution with the sorbent material. However, almost all biosorption studies reported so far have been based on single-metal solutions (Rudd et al., 1983; Forster, 1985; Swalaha, 1993 and Chu et al., 1997). Even when the influence of the second metal is examined, the results cannot be extrapolated and no predictive conclusions can be drawn (Chong and Volesky, 1995).

Moreover, the fundamentally inappropriate procedure has been to report the effect of the second metal based on its initial concentration. This is flawed since the biosorbent is really exposed to the initial concentrations for only a very brief period of time. It is important therefore to know final concentrations when dealing with equilibrium steady state.

The objectives of this study were to determine metal adsorptive capabilities of polysaccharides. The work reported here was undertaken in order to investigate the removal of metal by binding, individually and in combination, to extracellular polysaccharides and the effect of metal concentration on metal-EPS adsorption.

The application of the constituents of activated sludge biomass, such as polysaccharides, as adsorbents for the removal of heavy metals from contaminated water will contribute toward opening a novel area of biotechnology.

5.2 HYPOTHESIS

Having noted that activated sludge extracellular polysaccharides contain anionic groups it was hypothesized that they are capable of adsorbing metals from solution.

5.3 AIMS

- a) To determine the adsorptive capabilities of the purified EPS to metal ions,
- b) To determine the mechanisms of adsorption of metals to polysaccharide, and
- c) To determine the selectivity of EPS to metal uptake.

5.4 OBJECTIVES

- a) To determine the adsorptive capability of domestic and industrial sludge EPS to six metal species, Zn^{2+} , Ni^{2+} , Cu^{2+} , Cd^{2+} , Cr^{3+} , and Cr^{6+} , at varying concentration, and
- b) To plot adsorption isotherms and thereby determine the mechanism/s of polysaccharide-metal interactions.

5.5 MATERIALS AND METHODS

5.5.1 Pretreatment of materials

Polypropylene tubes, vials and all glassware were soaked overnight, at ambient temperature in 5% (v/v) Extran MA-01 alkaline. Vials and centrifuge tubes were then rinsed in triple deionised water and subsequently dried. Glassware, including pipettes, were subjected to a stringent acid wash procedure, i.e., rinsing in 50% (v/v) nitric acid (HNO_3), tap water followed by 50% hydrochloric acid, tap water and finally, rinsing in triple deionised water with subsequent drying (Smith and Vasiloudis, 1989).

5.5.2 Preparation of solutions

Heavy metal species used for batch experimentation included Cu^{2+} , Cd^{2+} , Zn^{2+} , Ni^{2+} , Cr^{3+} , and Cr^{6+} . Analar-grade glassware was used throughout for preparation of metal solutions. Aqueous metal stock solutions of 1000 mg.l^{-1} were prepared in one litre volumes and stored at 4°C in volumetric flasks. Working solutions of each metal were prepared by allowing aliquots of stock solution to attain room temperature before preparing required concentrations. Chemicals, $\text{K}_2\text{Cr}_2\text{O}_7$, $\text{CrK}(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$, $\text{NiCl}_2 \cdot 12\text{H}_2\text{O}$, $\text{Cu}(\text{NO}_3)_2 \cdot 3\text{H}_2\text{O}$, $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ and $\text{Cd}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ were analar-grade.

Heavy metal species used in the experiments were prepared individually from 1000 mg. ℓ^{-1} stock solutions when required. Working solutions of 31.25, 62.50, 93.75 and 125.00 mg. ℓ^{-1} of the Cr^{6+} , Cr^{3+} , Ni^{2+} , Cu^{2+} , Cd^{2+} , Zn^{2+} were prepared from stock solutions. Experiments were performed per single metal solution in triplicate. Aliquots of 8 ml metal solutions were dispensed into a propylene centrifuge tubes. These were exposed to 2 ml quantities of each domestic and industrial EPS sample. The final reaction concentrations of metal-ions were 25.00, 50.00, 75.00 and 100.00 mg. ℓ^{-1} .

A dialysis sac containing 2 ml deionised water was inserted into each centrifuge tube. Controls of each concentration of metal solution were prepared with 2ml deionised water substituted for EPS solution. Tubes were incubated upright at 25°C for 5 h at 150 rpm in an orbital shaker. After incubation, 2 ml was removed by pipette and diluted. Metal concentrations in these samples were determined using flame atomic absorption spectrophotometry.

5.5.3 Biosorption in a Ni^{2+} - Cr^{6+} mixture

The method was similar to a single system adsorption except that metal solutions now contained combinations of Ni^{2+} - Cr^{6+} in the concentration range of 25 to 100 $\text{mg} \cdot \ell^{-1}$ of each one of the metals. The final metal concentration C_f in the test solutions were determined by Atomic Adsorption Spectrophotometry, leading to the respective calculated values for biosorbent metal uptakes q_1 and q_2 for the first metal and for the second metal in the sorption system using the general definition (Trujillo et al, 1991):

$$q(\text{mmol/g}) = V(C_i - C_f)/m$$

where C_i is the initial metal concentration in solution of volume V , and m is the freeze-dried mass of the EPS used. Appropriate controls and blanks were examined throughout the sorption experiments to check glassware sorption of metals and other potential side effects.

5.5.4 Analysis of data

Data was converted to ratios of quantities metal biosorbed ($\text{mg} \cdot \text{g}^{-1}$) versus amount of EPS, percentage metal biosorbed plotted against metal ion concentration ($\text{mg} \cdot \ell^{-1}$). These were plotted as histograms. Data was also plotted as adsorption isotherms

according to the Langmuir Model in the form of metal concentration bound in mg.g^{-1} versus metal concentration in free solution in mg.l^{-1} . Data was linearised with the transformation $1/q_e$ vs C to determine the goodness of fit test to the Langmuir Model.

5.6 RESULTS

5.6.1 Biosorption in a single metal solution

Zinc biosorption, by both industrial and domestic sludge polysaccharides, demonstrated decreasing biosorption quantities at a concentration lower than $75 \text{ mg.}\ell^{-1}$ with decreasing percentage biosorption as zinc concentration in solution increases (Figs. 5.2 and 5.3). Quantities of zinc adsorbed and percentages removed were higher for domestic EPS than for industrial EPS indicating greater zinc binding capacity for domestic EPS. Zinc adsorption by domestic EPS decreased as metal concentration increased in the range 0-75 $\text{mg.}\ell^{-1}$ whilst at 100 $\text{mg.}\ell^{-1}$ adsorption showed a distinct change with quantities adsorbed being greater than at any other concentration level. The highest percentage biosorbed was at 25 $\text{mg.}\ell^{-1}$ for both types of polysaccharides. This is an indication of a fixed number of available sites for adsorption. An increase in the quantities in solution for biosorption at a concentration which is less than 75 $\text{mg.}\ell^{-1}$ would thus not proportionately increase biosorption of the zinc in solution.

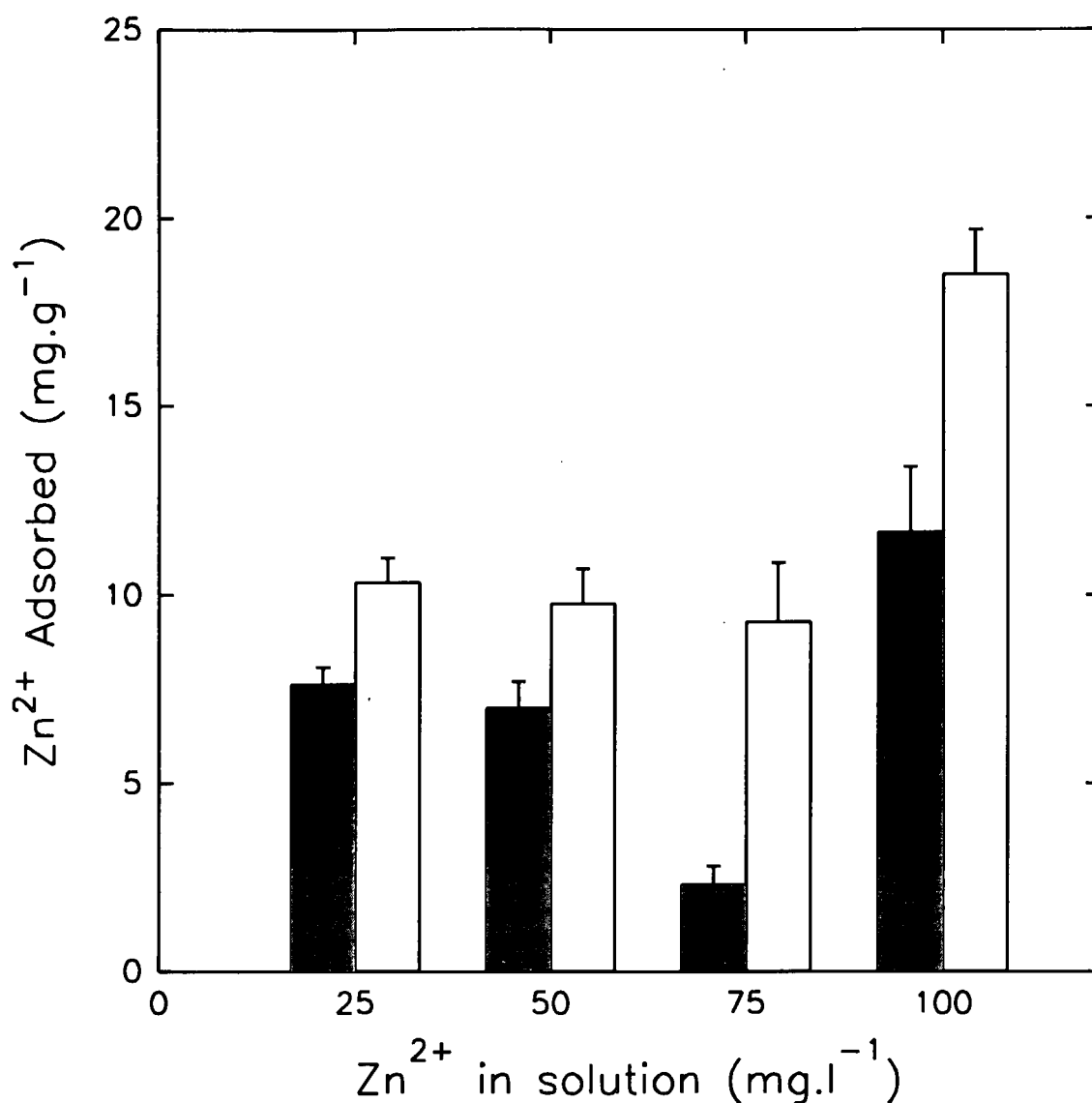


FIGURE 5.2. Quantities of zinc adsorbed by industrial (■) and domestic (□) sludge polysaccharides after incubation with solutions of increasing metal-ion concentration. Histograms represent mean values of triplicates. Error bars represent deviation of the results from the mean.

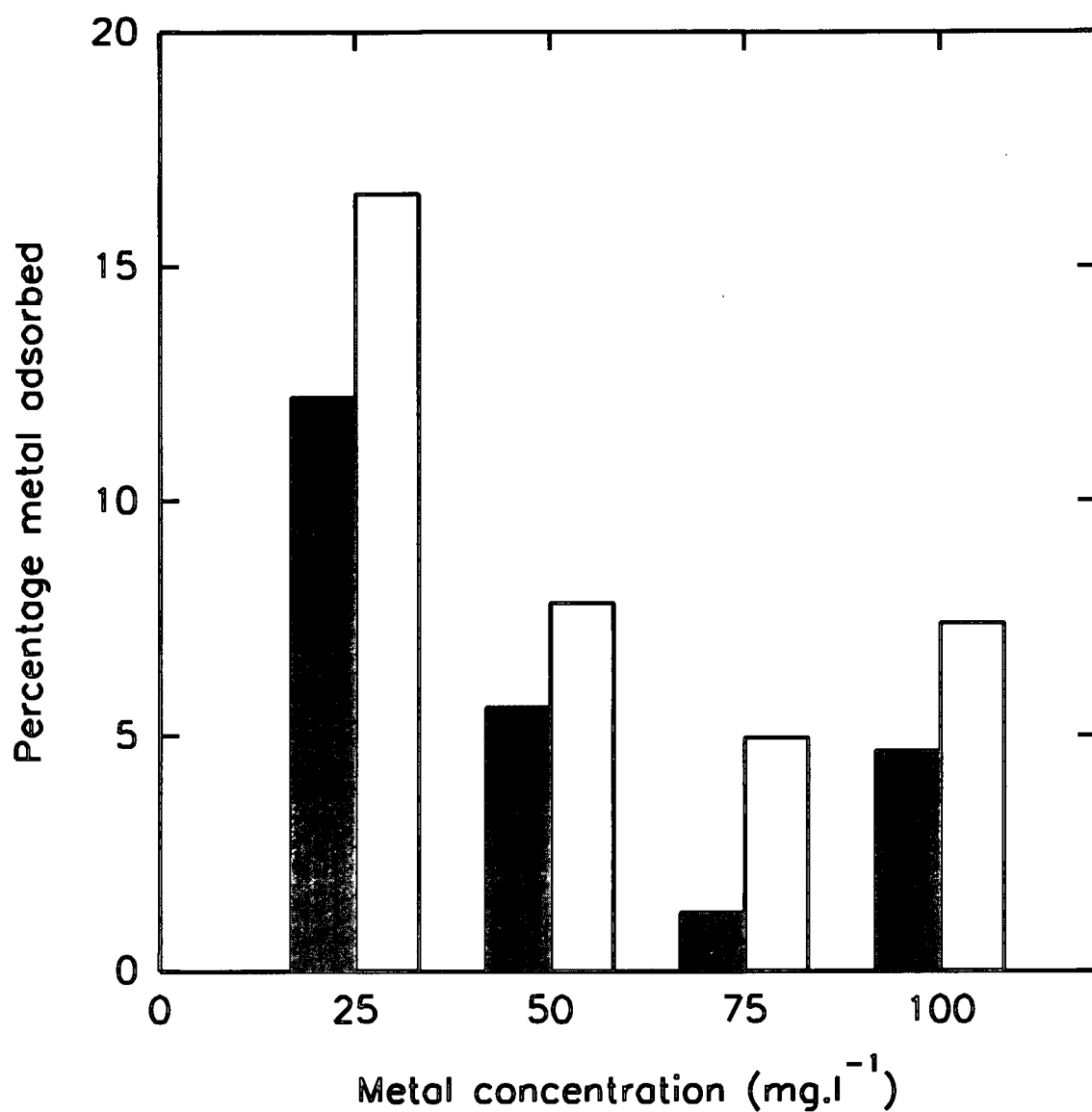


FIGURE 5.3. Percentage adsorption of zinc by industrial (■) and domestic (□) sludge polysaccharides after incubation with solutions of increasing metal-ion concentration.

Quantities of nickel adsorbed by both domestic and industrial polysaccharides increased as nickel concentration in solution increased (Fig. 5.4). Domestic polysaccharide adsorption of nickel reached an optimum at 75 mg.l^{-1} . This was also similar to industrial polysaccharide adsorption of nickel. Percentage adsorption of nickel by both polysaccharides increased as concentration of nickel in solution increased (Fig. 5.5). This indicates an increase in the availability of binding sites for nickel at a concentration which is less than 75 mg.l^{-1} . There were significant differences observed in the adsorption of nickel between domestic EPS and industrial EPS. At 25 mg.l^{-1} more nickel was adsorbed by industrial polysaccharides than domestic polysaccharides. This was an exception to all other concentration levels where the opposite happened.

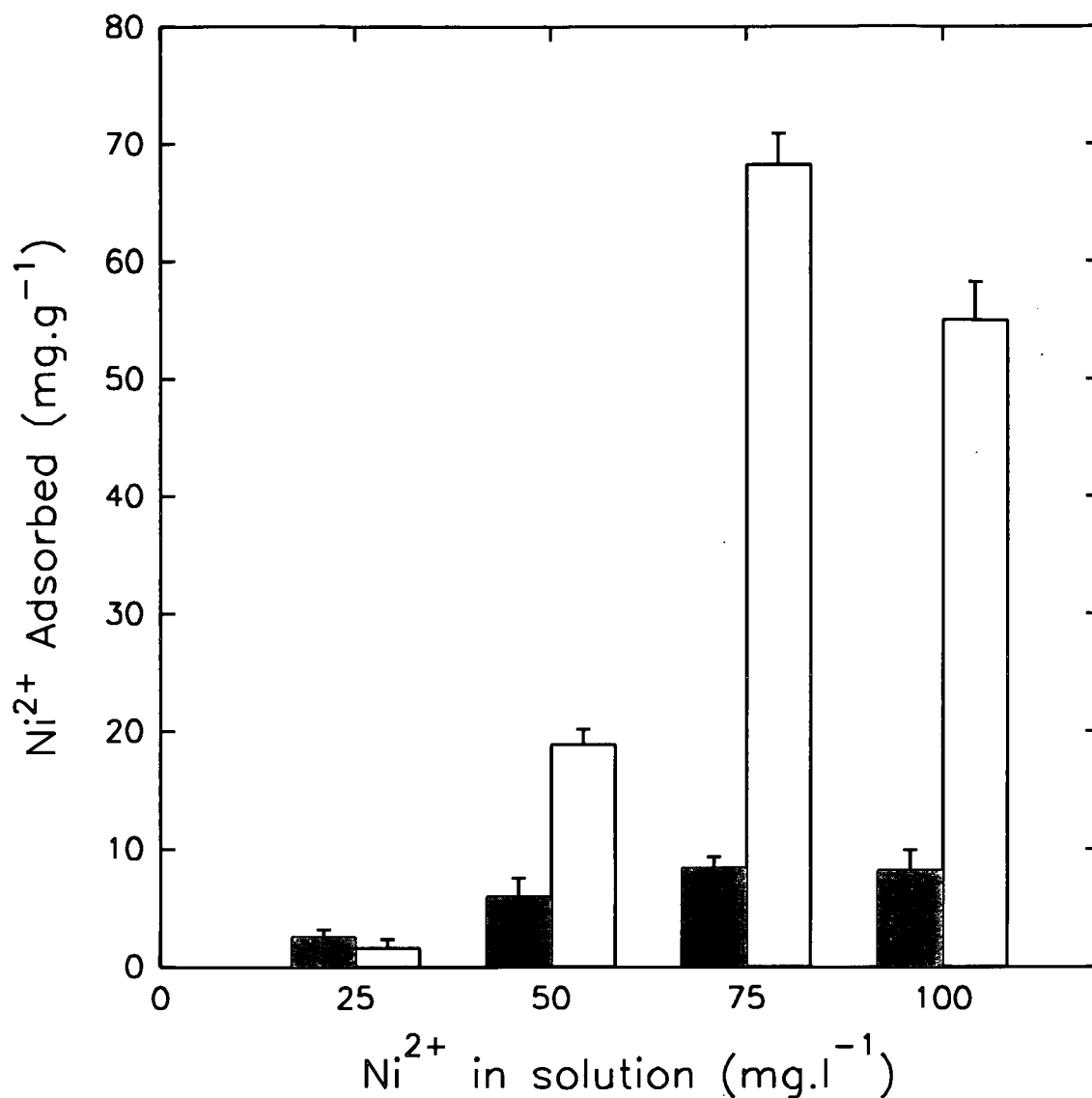


FIGURE 5.4 Quantities of nickel adsorbed by industrial (■) and domestic (□) sludge polysaccharides after incubation with solutions of increasing metal-ion concentration. Histograms represent mean values of triplicates. Error bars represent deviation of the results from the mean.

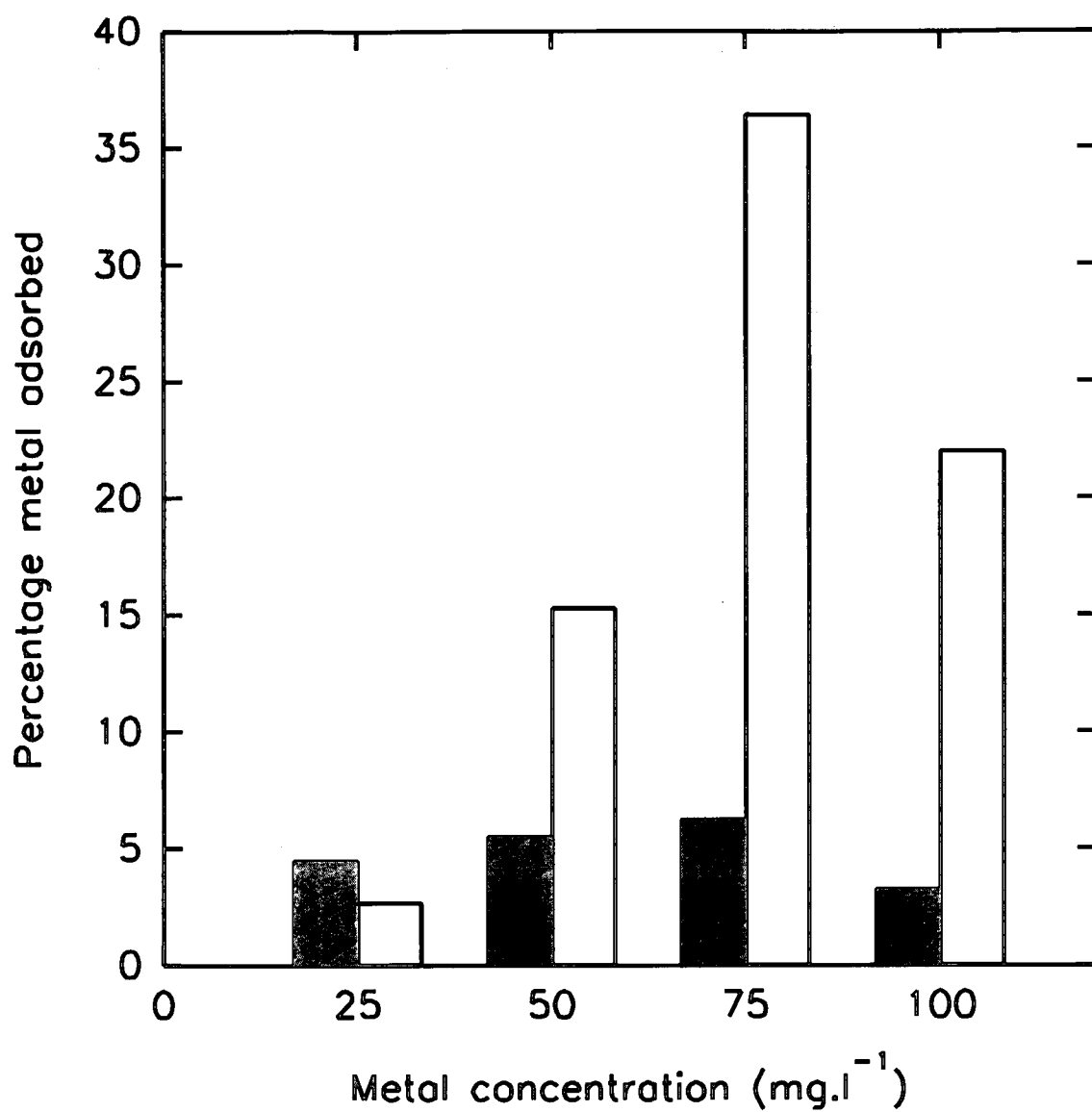


FIGURE 5.5. Percentage adsorption of nickel by industrial (■) and domestic (□) sludge polysaccharides after incubation with solutions of increasing metal-ion concentration.

The trend in the quantities of Cu adsorbed by domestic and industrial sludge EPS was similar to the adsorption of nickel. There was an increase in adsorption between 25-75 mg. ℓ^{-1} range. Optimum adsorption concentration was at 75 mg. ℓ^{-1} (Fig. 5.6). Percentage adsorption did not develop any clear pattern except that in all concentration levels percentage adsorption was greater for domestic EPS than for industrial EPS (Fig. 5.7).

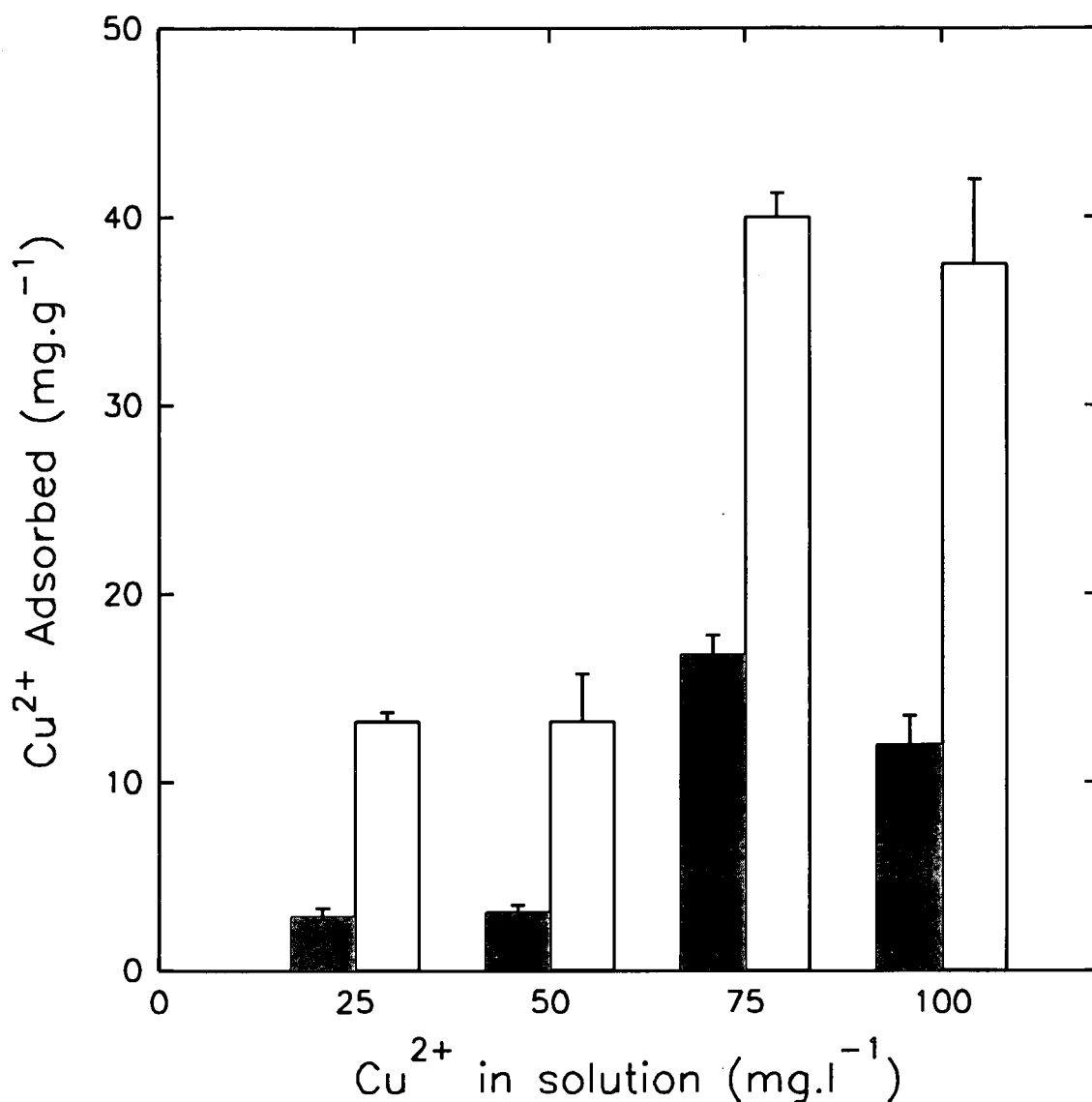


FIGURE 5.6. Quantities of copper adsorbed by industrial (■) and domestic (□) sludge polysaccharides after incubation with solutions of increasing metal-ion concentration. Histograms represent mean values of triplicates. Error bars represent deviation of the results from the mean.

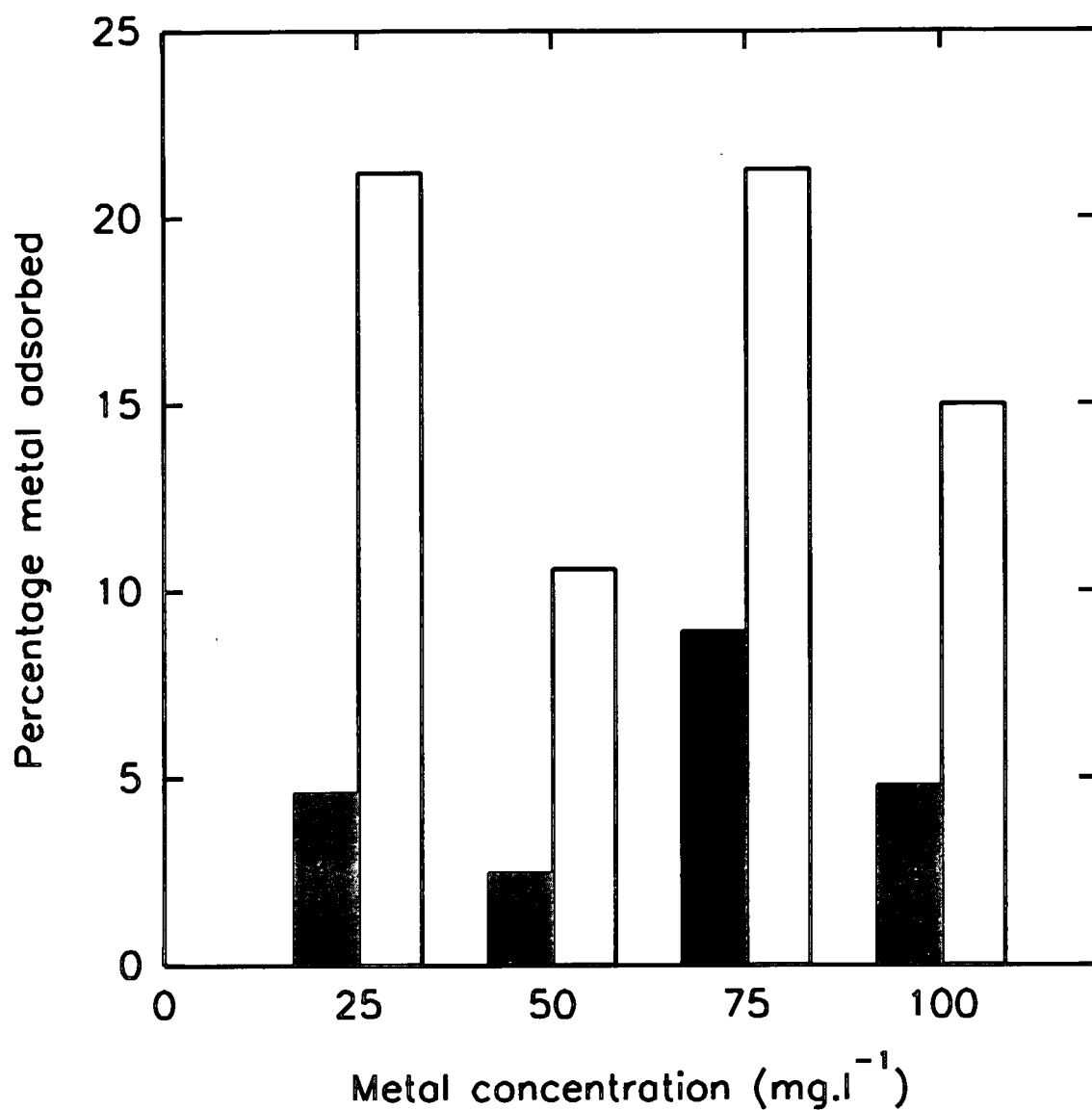


FIGURE 5.7. Percentage adsorption of copper by industrial (■) and domestic (□) sludge polysaccharides after incubation with solutions of increasing metal-ion concentration.

Quantities of cadmium adsorbed decreased as metal concentration increases from 25 to 75 $\text{mg} \cdot \ell^{-1}$ (Fig. 5.8). More cadmium was adsorbed at 100 $\text{mg} \cdot \ell^{-1}$ than at any other metal concentration level. Domestic EPS showed a more adsorption efficiency than industrial EPS at metal concentrations between 25 and 75 $\text{mg} \cdot \ell^{-1}$. Percentage metal adsorption decreased as cadmium in solution increased within the range 25-75 $\text{mg} \cdot \ell^{-1}$ and then there was an increase in metal adsorbed at 100 $\text{mg} \cdot \ell^{-1}$ (Fig. 5.9). Highest percentage of cadmium adsorbed by domestic polysaccharides was at 25 $\text{mg} \cdot \ell^{-1}$ and for industrial polysaccharides at 100 $\text{mg} \cdot \ell^{-1}$. This shows that at high concentrations more sites are available for binding of cadmium in the industrial sludge polysaccharides than in the domestic sludge polysaccharides.

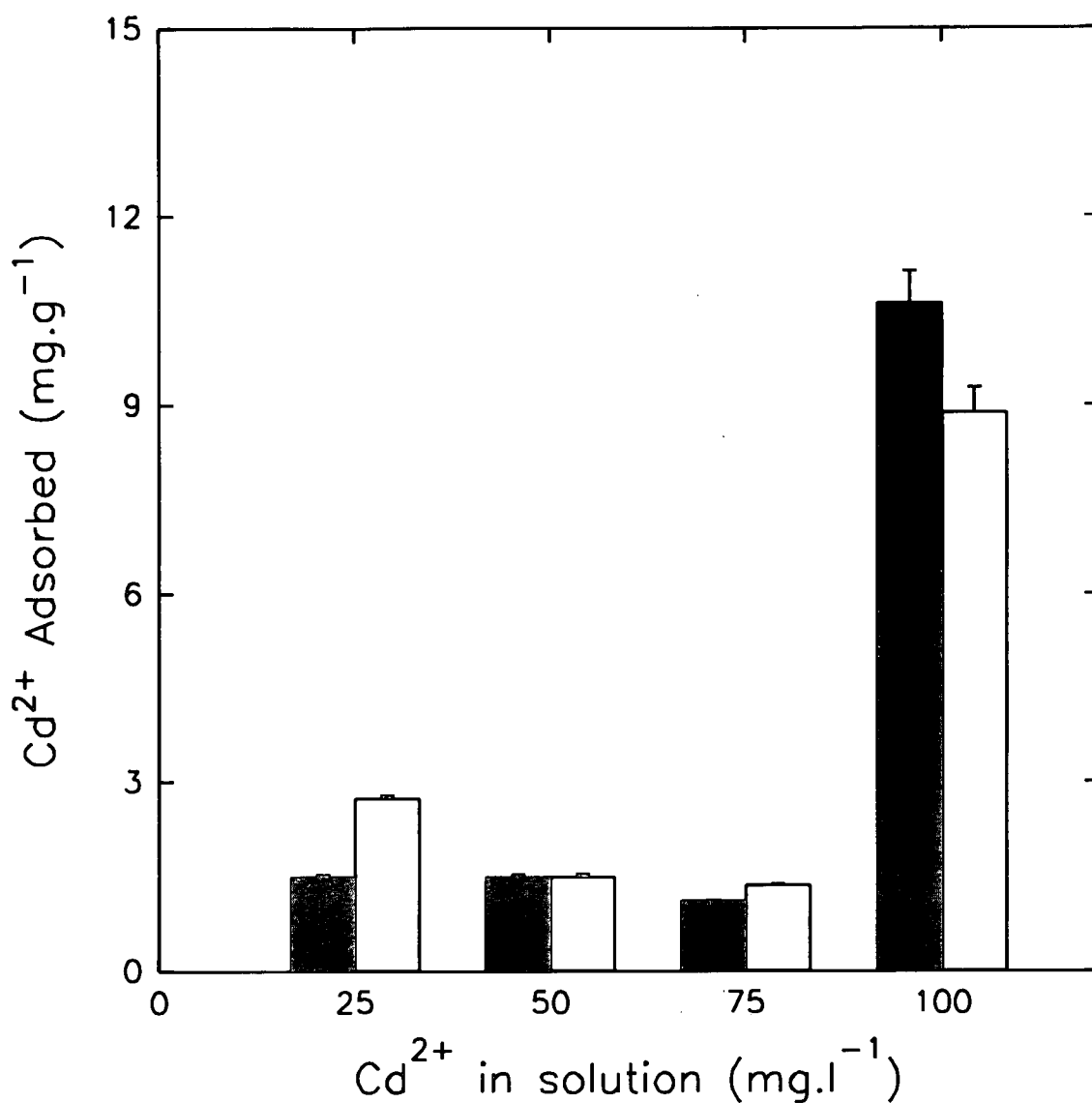


FIGURE 5.8. Quantities of cadmium adsorbed by industrial (■) and domestic (□) sludge polysaccharides after incubation with solutions of increasing metal-ion concentration. Histograms represent mean values of triplicates. Error bars represent deviation of the results from the mean.

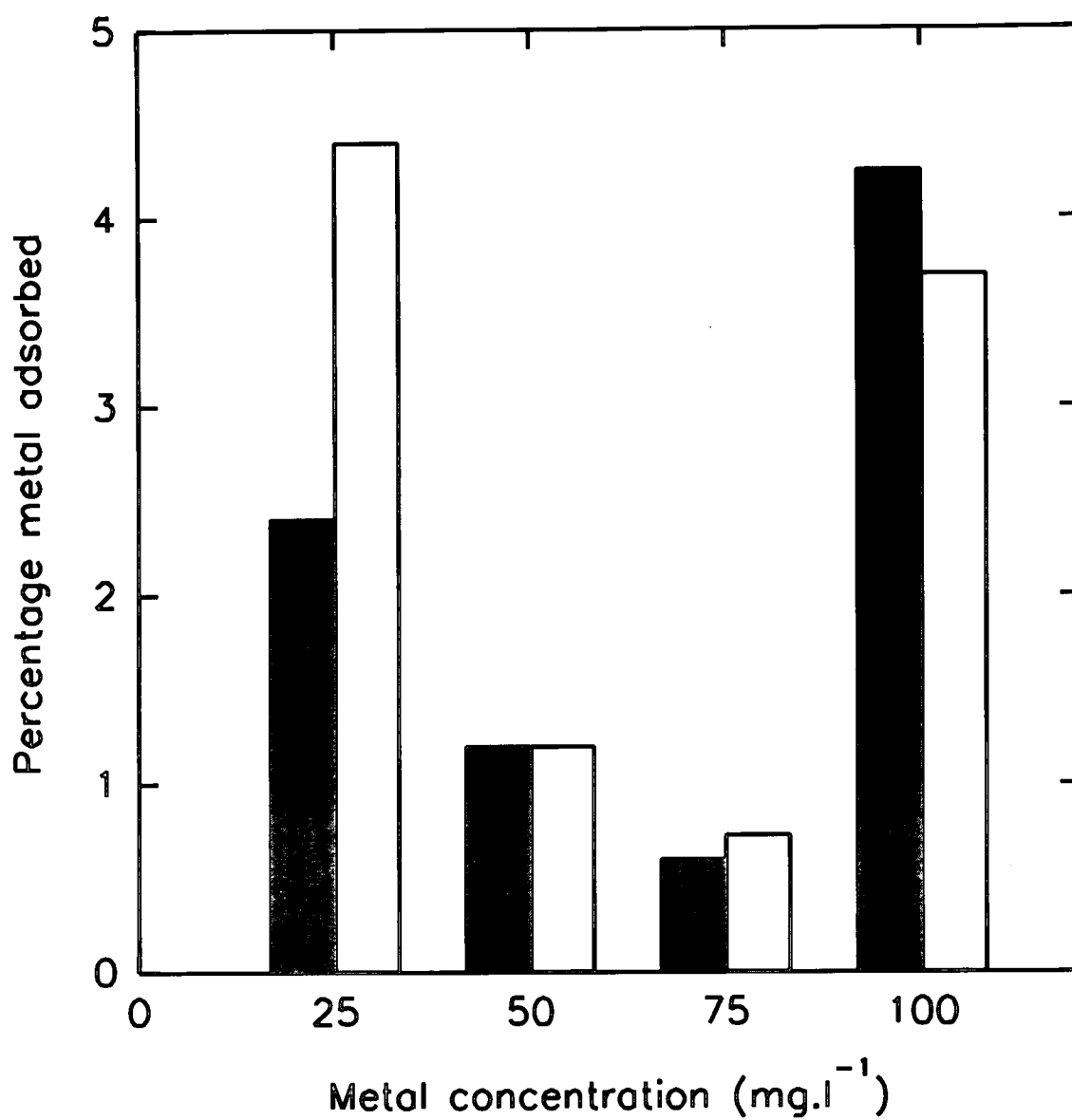


FIGURE 5.9. Percentage adsorption of cadmium by industrial (■) and domestic (□) sludge polysaccharides after incubation with solutions of increasing metal-ion concentration.

Quantities and percentage adsorption by domestic sludge polysaccharides of Cr^{3+} showed no definite trend with adsorption quantities and percentages increasing and decreasing at different concentration (Fig. 5.10 and 5.11). Industrial sludge polysaccharide adsorption of Cr^{6+} increased as concentration of the metal in solution increased and reached an optimum adsorption concentration at $75 \text{ mg.}\ell^{-1}$ (Fig. 5.12 and Fig. 5.13) Quantities of Cr^{3+} adsorbed by domestic EPS were greater than those adsorbed by industrial EPS at $25\text{-}75 \text{ mg.}\ell^{-1}$ and the trend reversed at $100 \text{ mg.}\ell^{-1}$. Percentage adsorption of Cr^{3+} decreased as metal concentration increased. There was an exception where metal adsorbed increased tremendously at $100 \text{ mg.}\ell^{-1}$ and was greater than at any other metal concentration and this was highly noticeable in the case of an industrial EPS. In general Cr^{3+} adsorption efficiency was greater than adsorption efficiency of Cr^{6+} .

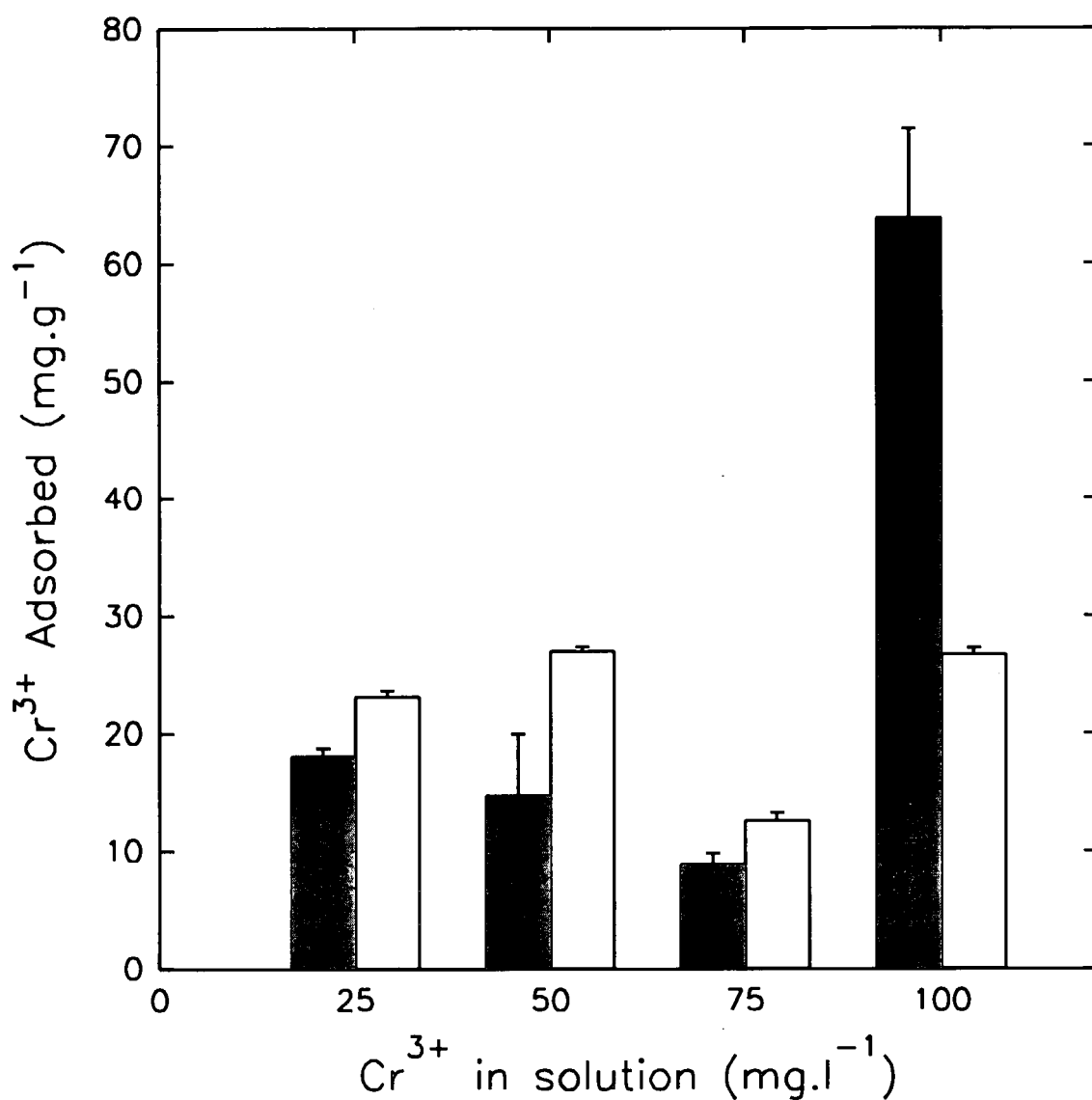


FIGURE 5.10. Quantities of Cr^{3+} adsorbed by industrial (■) and domestic (□) sludge polysaccharides after incubation with solutions of increasing metal-ion concentration. Histograms represent mean values of triplicates. Error bars represent deviation of the results from the mean.

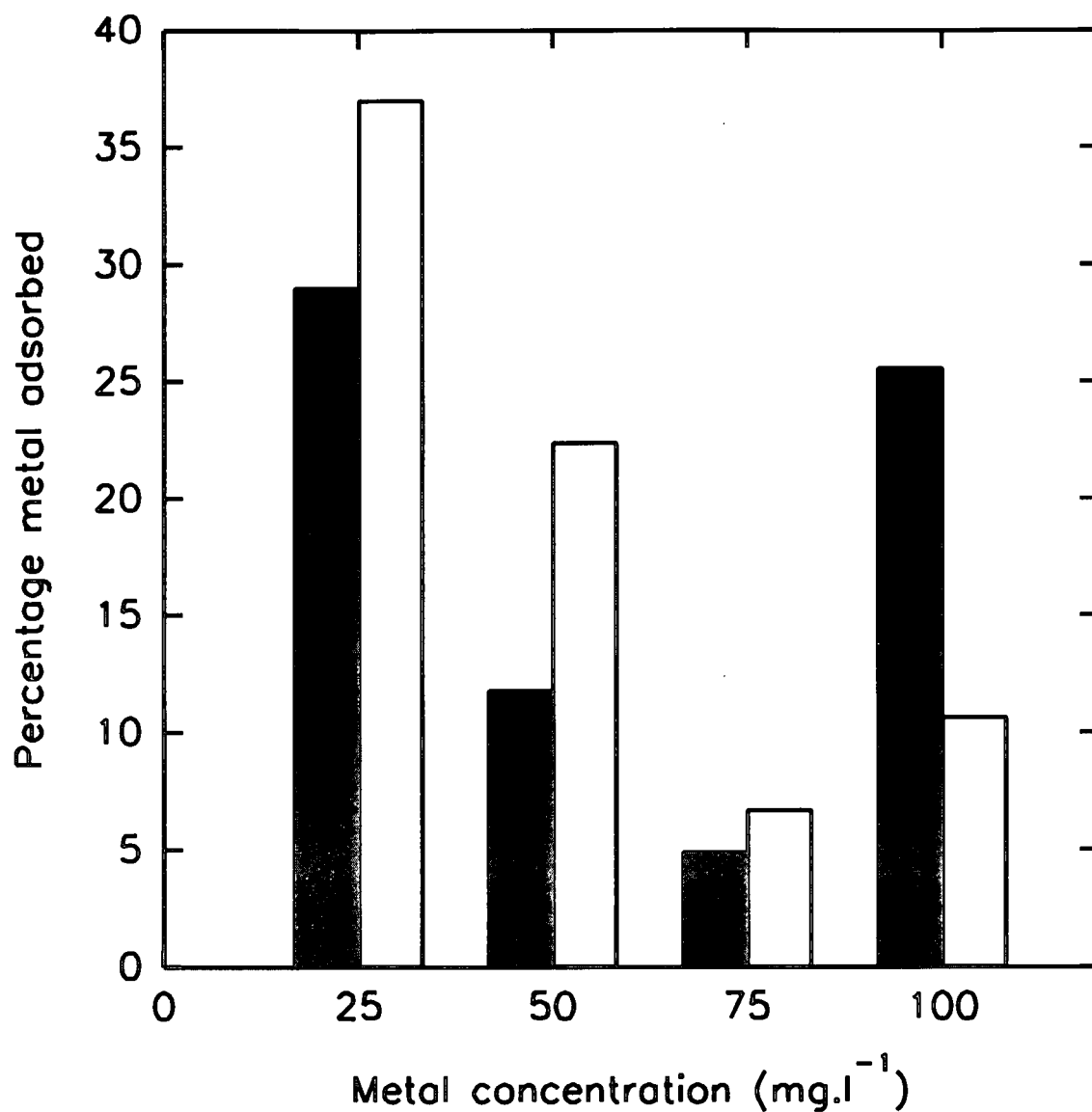


FIGURE 5.11. Percentage adsorption of Cr^{3+} adsorbed by industrial (■) and domestic (□) sludge polysaccharides after incubation with solutions of increasing metal-ion concentration.

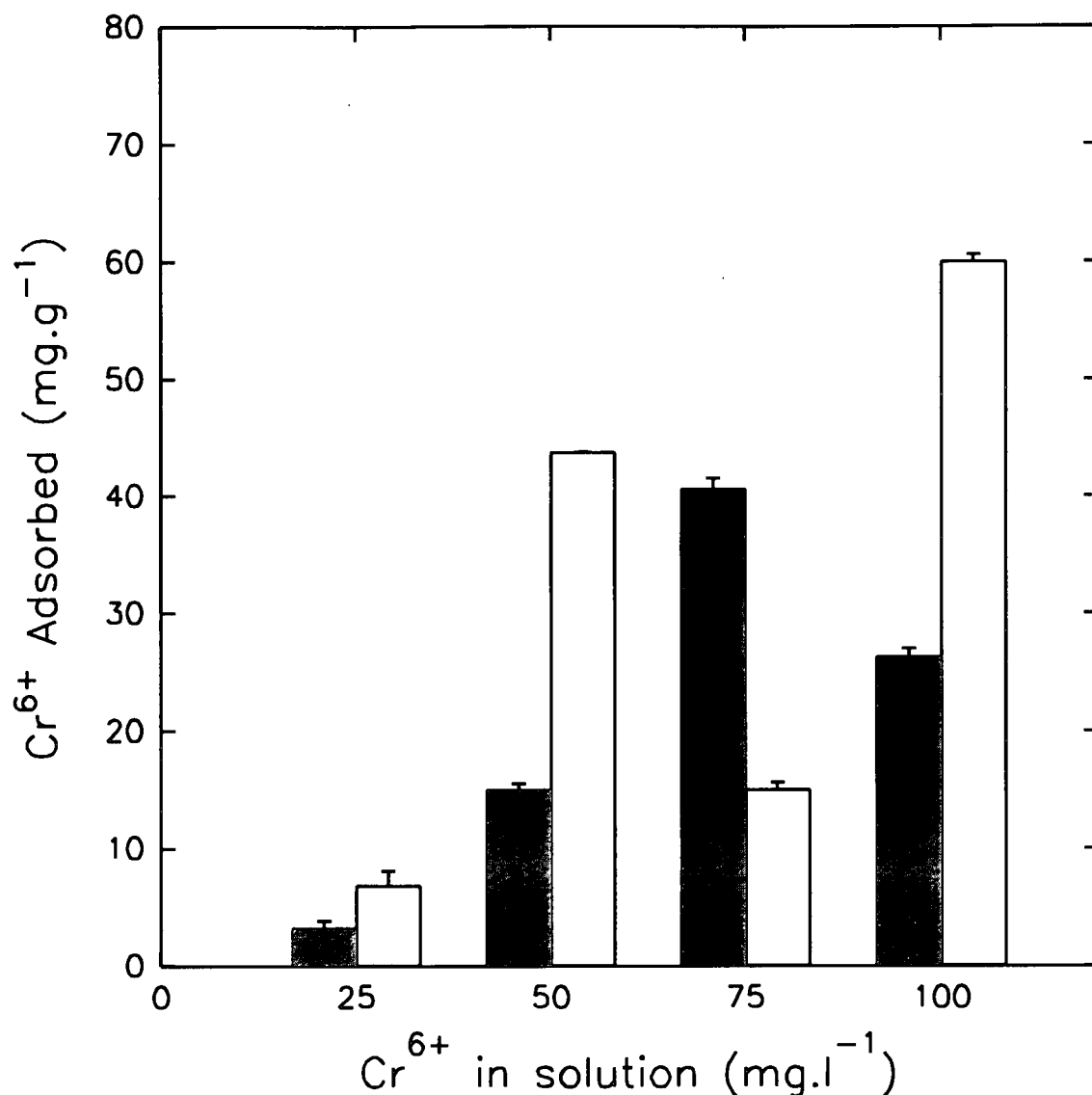


FIGURE 5.12. Quantities of Cr^{6+} adsorbed by industrial (■) and domestic (□) sludge polysaccharides after incubation with solutions of increasing metal-ion concentration. Histograms represent mean values of triplicates. Error bars represent deviation of the results from the mean.

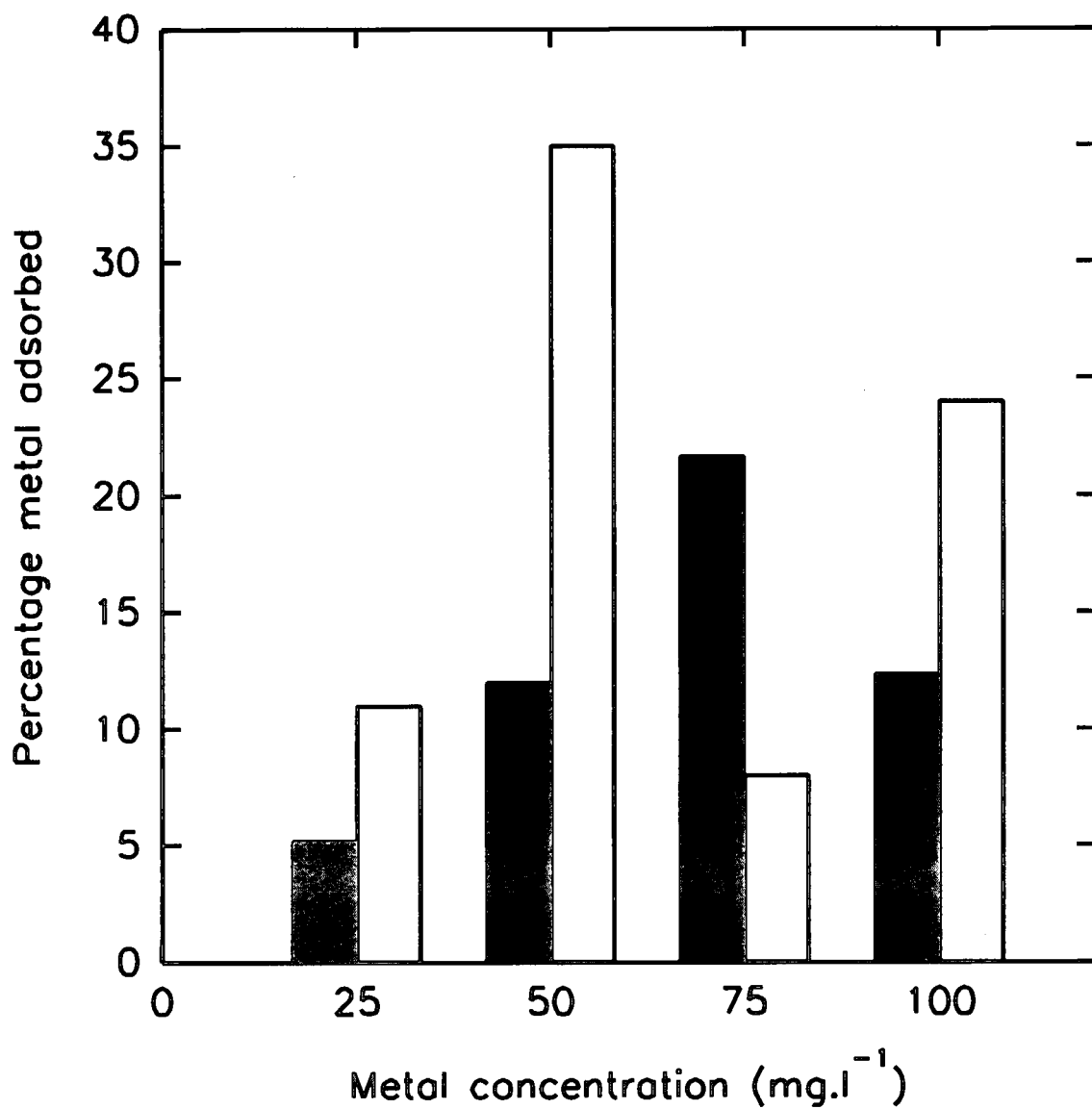


FIGURE 5.13. Percentage adsorption of Cr^{6+} by industrial (■) and domestic (□) sludge polysaccharides after incubation with solutions of increasing metal-ion concentration.

Comparison of different metals biosorbed at different metal concentrations produced interesting results. Adsorption of Cr^{3+} from solution was highest at $100 \text{ mg.}\ell^{-1}$ while Ni was the lowest metal biosorbed at $100 \text{ mg.}\ell^{-1}$ (Fig. 5.15). Cr^{6+} was the highest metal biosorbed at $75 \text{ mg.}\ell^{-1}$. As metal-ion quantities increased in solution an increase in adsorption percentage between $25 \text{ mg.}\ell^{-1}$ and $75 \text{ mg.}\ell^{-1}$ was noted (Fig. 5.14 and 5.15). A fixed site theory of adsorption developed by Ruthven (1984) can be applicable here. This means that a fixed number of sites available for metal adsorption were saturated at high metal concentration. Hence higher metal concentration did not increase efficiency of adsorption.

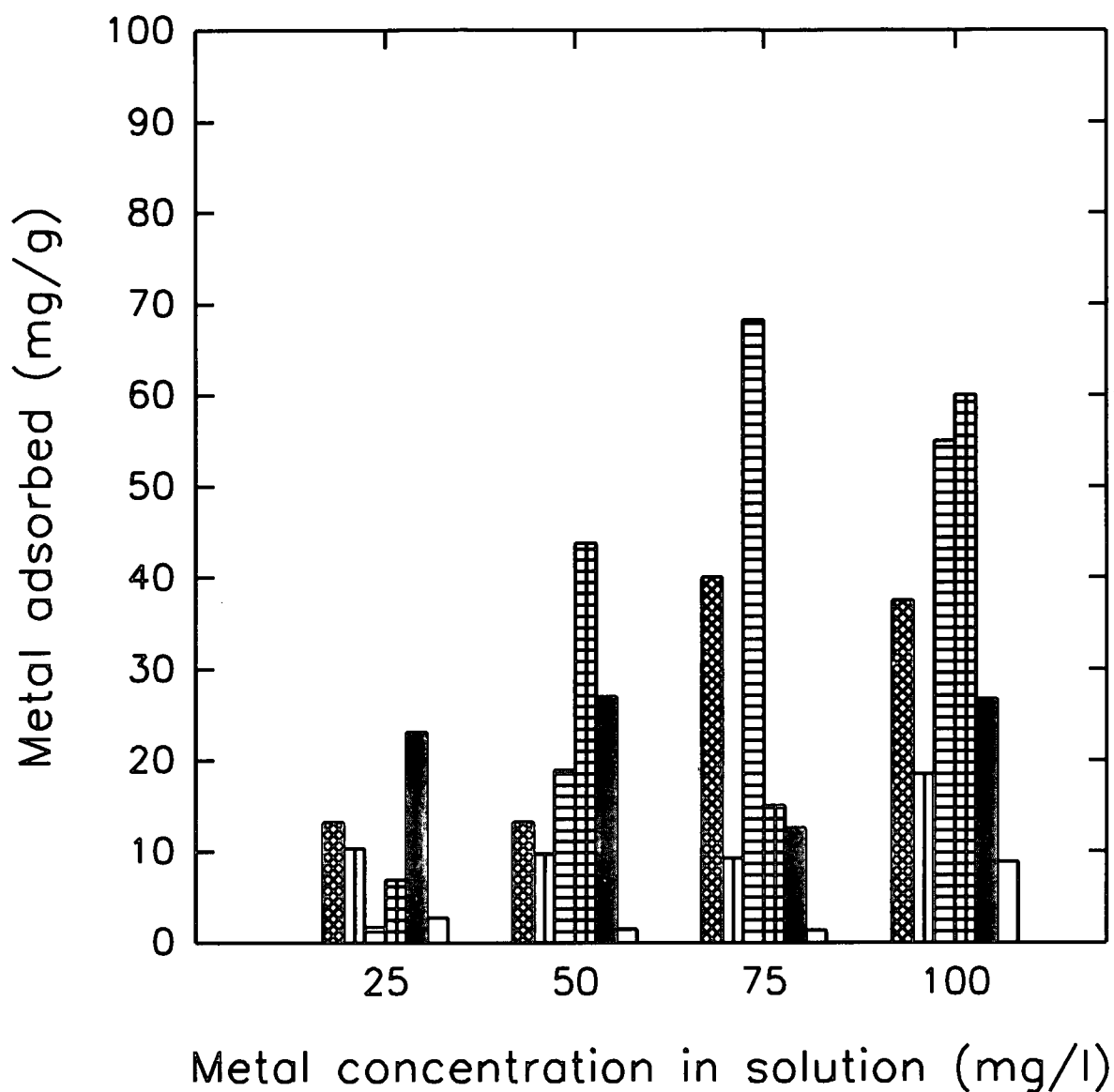


FIGURE 5.14 Comparison of the quantities of copper (▣), zinc (▤), nickel (▥), chromium(VI) (▦), chromium(III) (▧) and cadmium (▨) adsorbed by domestic sludge EPS at different metal concentration.

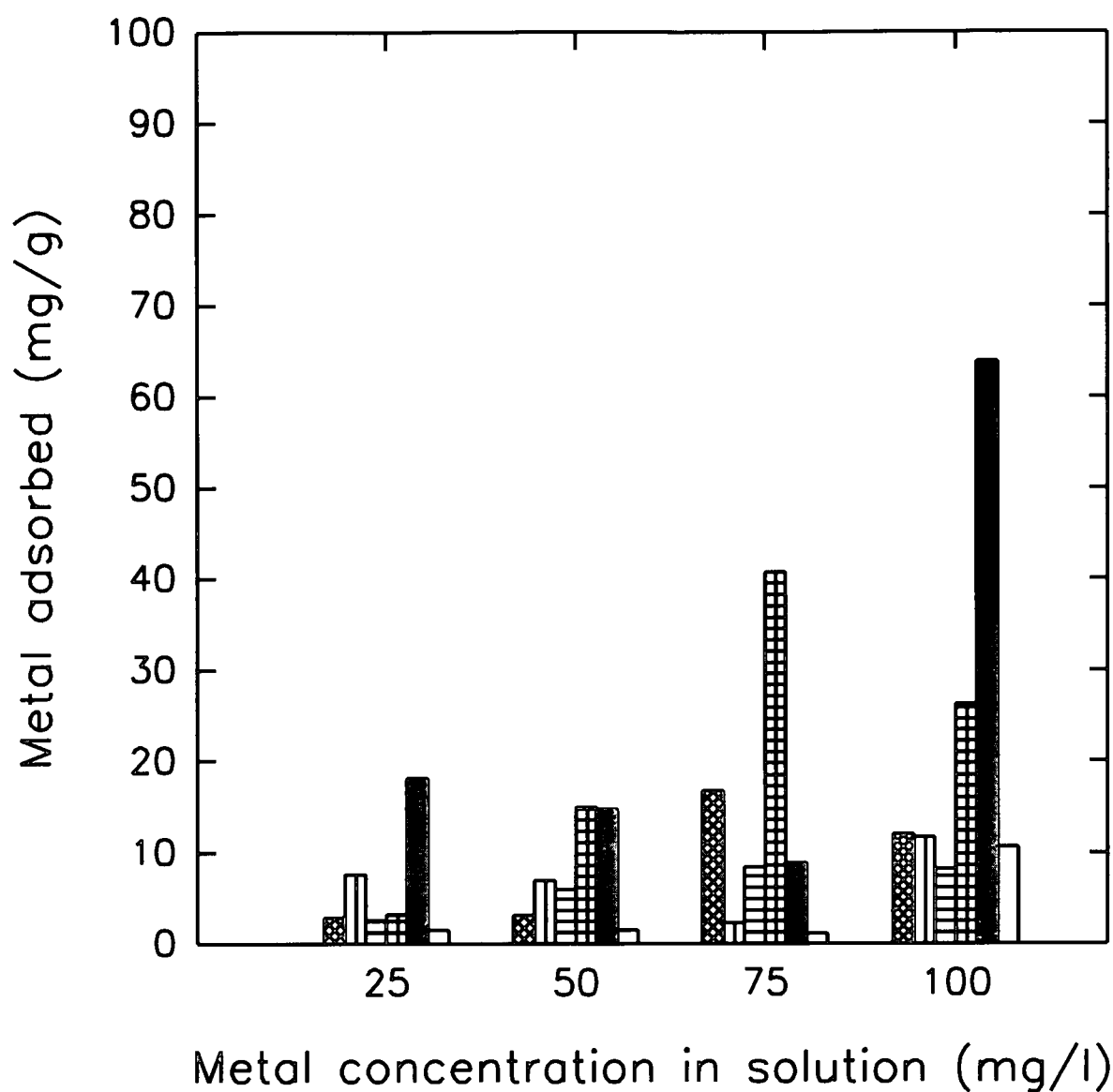


FIGURE 5.15. Comparison of the quantities of copper (▨), zinc (▧), nickel (▩), chromium(VI) (▦), chromium(III) (■) and cadmium (□) adsorbed by industrial sludge EPS at different metal concentration.

5.6.2 Construction of adsorption isotherms

To investigate mechanistic aspects of metal binding, adsorption isotherms of q_e versus C were constructed. The portion of the curves obtained indicate how the adsorption was classified. The steep slope of the initial portion of Langmuir isotherms, indicates a very high affinity at the lower metal concentration. Curves which are typical of the results obtained by the equilibrium analysis method are shown in Fig. 5.16 to Fig. 5.27. The Langmuir isotherms indicate that as sites on the polysaccharides become occupied, it becomes increasingly less likely for metal ions to encounter the few vacant sites.

Nickel indicated a Langmuir behaviour showing the attainment of the optimum adsorption concentration for both types of polysaccharides (Fig. 5.24). Cr^{6+} also showed the same behaviour (Fig. 5.20). Cadmium, zinc and Cr^{3+} showed the "BET" behaviour (Fig. 5.16, 5.18, 5.22, and 5.26). This is indicative of the competitive adsorption with intermolecular attraction between adsorbed species. Therefore, nickel and Cr^{6+} binds to a fixed number of sites available and no further adsorption could occur at higher metal concentration.

5.6.3 Evaluation of complexation behaviour

The data used to plot q_e against C was also used to plot $1/q_e$ versus $1/C$ to investigate further differences in complexation behaviour between the metals. For comparison, data for each metal were subjected to regression analysis for both the monophasic and biphasic model. The conditional stability constants for the complexes formed between the polysaccharides and the six metal tested and the adsorption capacity of the polysaccharides are listed in Table 5.1. Differences in the metal binding capacities of the domestic and industrial polysaccharides are apparent from these results.

Binding intensity of Cd to industrial EPS was the highest since K_a is the measure of the affinity of metals to binding sites (Table 5.1). This implies that even though the number of binding sites for Cd may be limited, it has a high binding capacity. Nickel showed the highest binding capacity for domestic EPS.

TABLE 5.1 : Determination of binding characteristics of industrial and domestic sludge polysaccharides.

BINDING CONSTANTS				
	Industrial EPS		Domestic EPS	
	K_a (l/mol)	X_m (mol/g)	K_a (l/mol)	X_m (mol/g)
Zn	-35.71	35.40	4.278	373.8
Ni	9.188	67.94	26.09	54.93
Cr ³⁺	-12.07	31.63	-3.126	22.68
Cr ⁶⁺	10.44	79.11	5.635	69.69
Cd	76.16	71.79	-396.8	44.59
Cu	13.37	145.8	2.262	326.2

Adsorption isotherms, according to the Langmuir model, were plotted for all sludge polysaccharides-metal interactions (Fig. 5.16 to Fig. 5.27). Only nickel and chromium (VI) displayed first order regression coefficients of values greater than 0.90, indicating its conformity with the Langmuir model (Fig. 5.21 and 5.25). Cadmium adsorption by both industrial and domestic sludge polysaccharide demonstrated a bilayer or Brunauer, Emmet and Teller (BET) type adsorption isotherm (Fig. 5.16). It presented a linear regression coefficients of 0.35 for industrial EPS and 0.13 for domestic EPS thus nonconforming to the Langmuir model.

Trivalent chromium adsorption confirmed to the "BET" model and displayed a typical "S" shaped isotherm (Fig. 5.18). Both domestic and industrial sludge EPS present very low regression coefficients of 0.07 and 0.08 respectively.

Hexavalent chromium adsorption showed a difference in adsorption efficiency between industrial sludge polysaccharides and domestic sludge polysaccharides. The shape of the industrial sludge EPS isotherm appeared to fit the typical "L" shaped isotherms and had a regression coefficient of 0.90. The shape of the domestic sludge EPS isotherm did not show any trend and had a very low regression coefficient of 0.62.

The result of copper displayed a typical Langmuir type isotherms

(Fig. 5.22) although the regression analysis confirmed that it does not fit to the model with coefficients of 0.77 for industrial sludge EPS and 0.69 for domestic sludge EPS.

Nickel adsorption by both industrial sludge EPS and domestic sludge EPS displayed results which fit the Langmuir isotherm model. Both EPS types adsorption produced regression values which were greater than 0.90 and the graph showed a typical "L" shaped isotherm (Fig. 5.24). This indicates that only one type of binding sites on domestic EPS adsorbed nickel from solution. These binding sites could be charged carboxyl groups which exist on amino acid and glucuronic acid which was shown to be a component of domestic EPS.

Zinc adsorption by both domestic and industrial sludge polysaccharides displayed a "BET" relationship with regression coefficients of 0.42 and 0.017 respectively (Fig. 5.26).

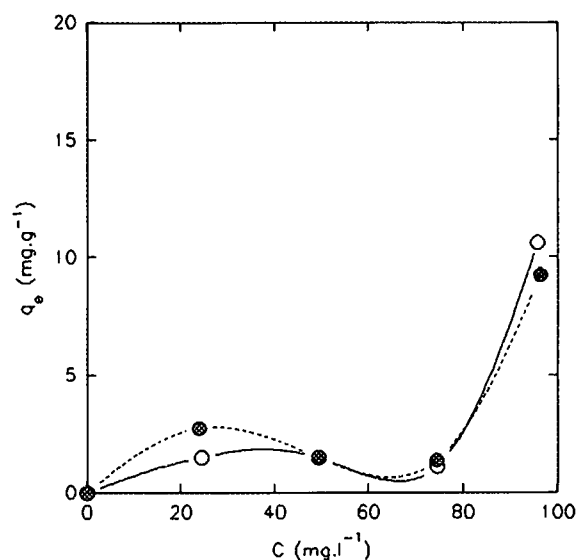


FIGURE 5.16. Langmuir adsorption isotherm of Cd^{2+} uptake (q_e) from solution by industrial (○) and domestic (●) sludge polysaccharides at varying equilibrium concentration C .

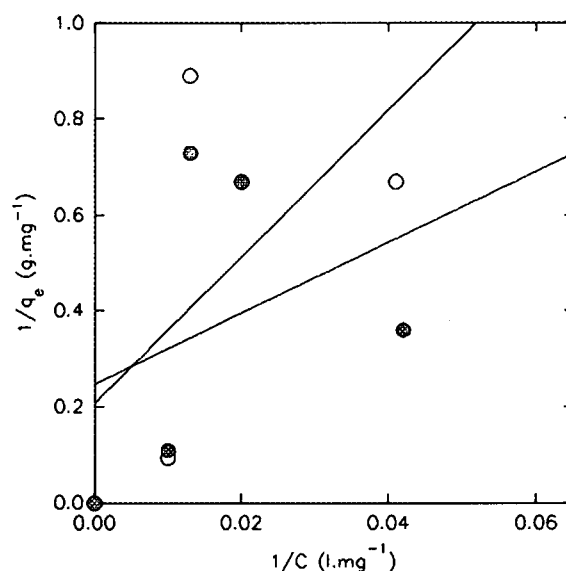


FIGURE 5.17. Reciprocal Langmuir plots for Cd^{2+} adsorption to industrial (○) and domestic (●) polysaccharides. Square of correlation coefficient for linearisation of isotherm is 0.10 for industrial sludge EPS and 0.13 for domestic sludge EPS.

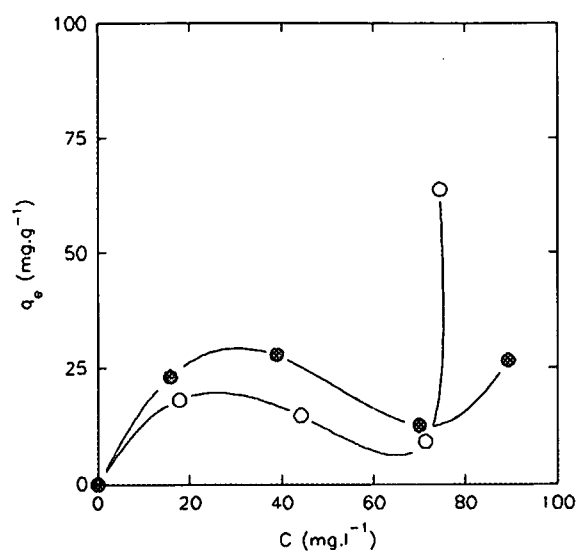


FIGURE 5.18. Langmuir adsorption isotherm of Cr^{3+} uptake (q_e) from solution to industrial (○) and domestic (●) sludge polysaccharides at varying equilibrium concentration C .

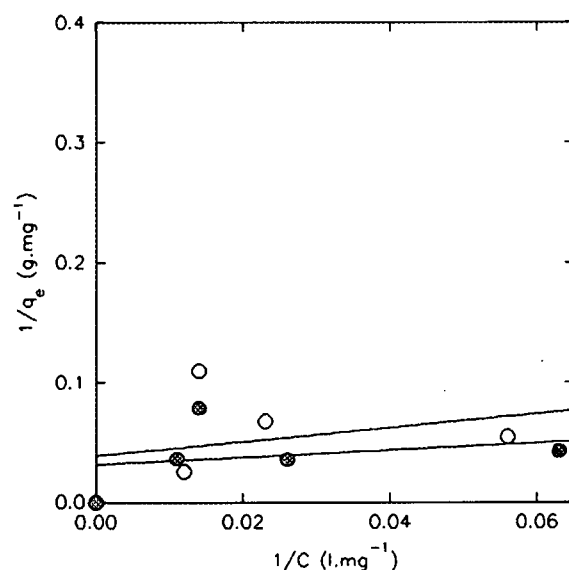


FIGURE 5.19. Reciprocal Langmuir plots for Cr^{3+} adsorption to industrial (○) and domestic (●) sludge polysaccharides.

Square of correlation coefficient for linearisation of isotherm is 0.08 for industrial sludge EPS and 0.07 for domestic sludge EPS.

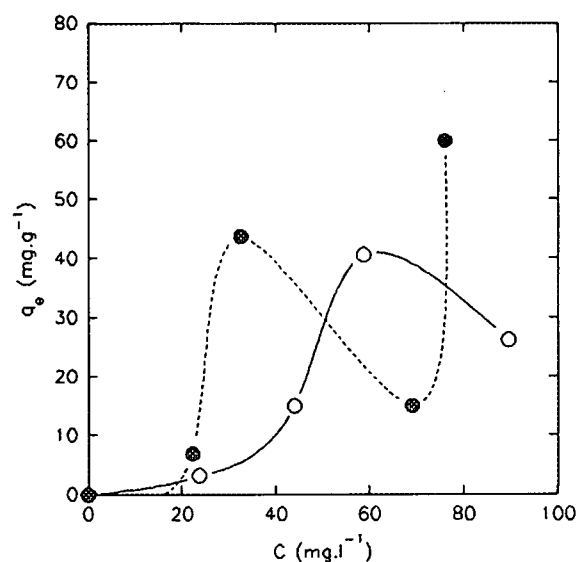


FIGURE 5.20. Langmuir adsorption isotherm of Cr^{6+} uptake (q_e) from solution by industrial (○) and domestic (●) sludge polysaccharides at varying equilibrium concentration C .

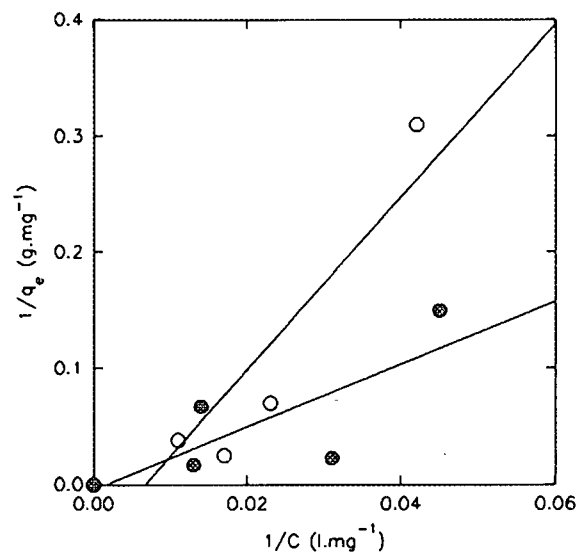


FIGURE 5.21. Reciprocal Langmuir plots for Cr^{6+} adsorption to industrial (○) and domestic (●) sludge polysaccharides. Square of correlation coefficient for linearisation of isotherm is 0.91 for industrial sludge EPS and 0.91 for domestic sludge EPS.

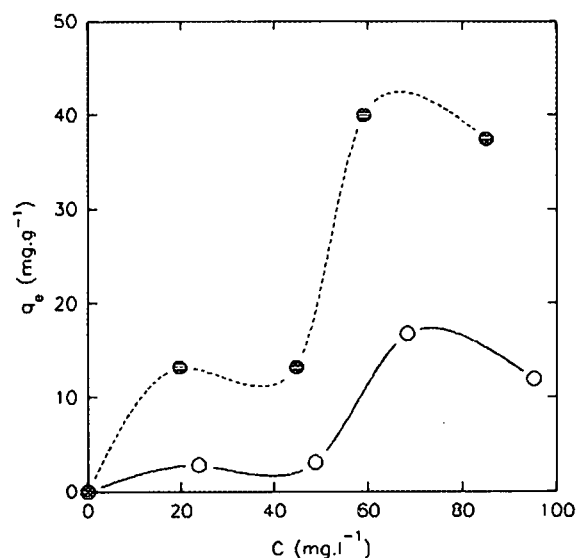


FIGURE 5.22 Langmuir adsorption isotherm of Cu^{2+} uptake (q_e) from solution to industrial (○) and domestic (●) sludge polysaccharides at varying equilibrium concentration C .

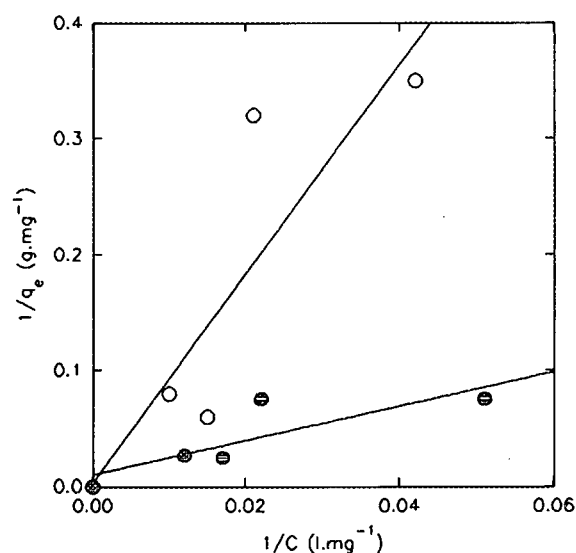


FIGURE 5.23. Reciprocal Langmuir plots for Cu^{2+} adsorption to industrial (○) and domestic (●) sludge polysaccharides. Square of correlation coefficient for linearisation of isotherm is 0.52 for industrial sludge EPS and 0.67 for domestic sludge EPS.

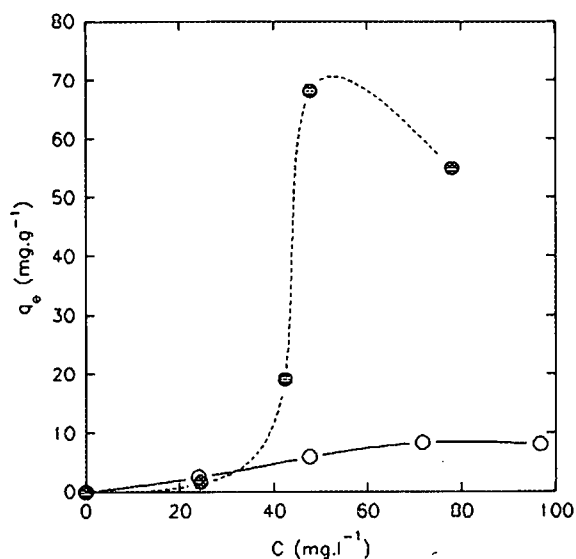


FIGURE 5.24. Langmuir adsorption isotherm of Ni^{2+} uptake (q_e) from solution by industrial (○) and domestic (●) sludge polysaccharides at varying equilibrium concentration C .

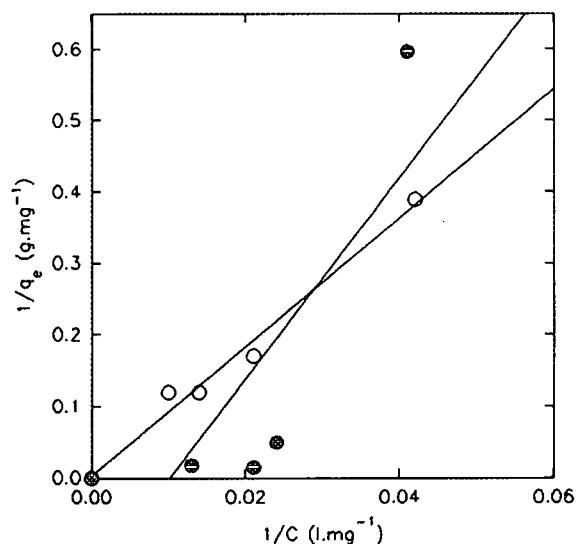


FIGURE 5.25. Reciprocal Langmuir plots for Ni^{2+} adsorption to industrial (○) and domestic (●) polysaccharides. Square of correlation coefficient for linearisation of isotherm is 0.97 for industrial sludge EPS and 0.87 for domestic sludge EPS.

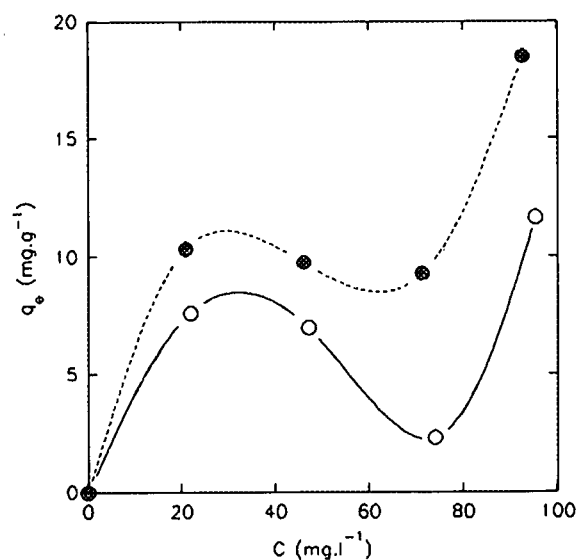


FIGURE 5.26. Langmuir adsorption isotherm of Zn^{2+} uptake (q_e) from solution by industrial (○) and domestic (●) sludge polysaccharides at varying equilibrium concentration C .

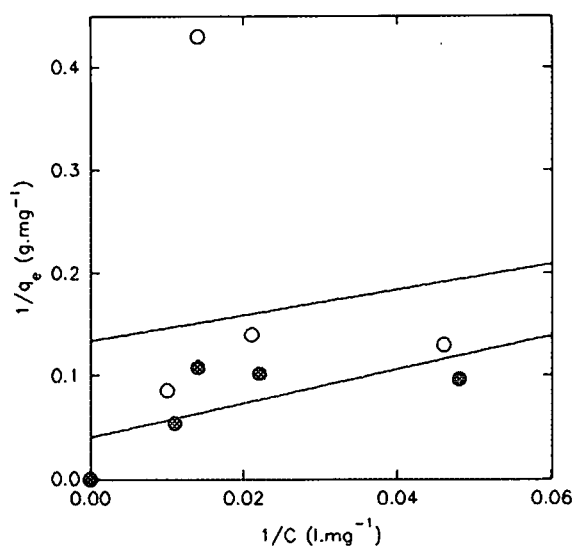


FIGURE 5.27. Reciprocal Langmuir plots for Zn^{2+} adsorption industrial (○) and domestic (●) sludge polysaccharides. Square of correlation coefficient for linearisation of isotherm is 0.07 for industrial sludge EPS and 0.12 for domestic sludge EPS.

It was imperative to investigate the relevance of the data used for Langmuir model to Freundlich model (Fig. 5.28 to Fig. 5.33). This was achieved by plotting Freundlich adsorption isotherms of $\log q_e$ Vs $\log C$. The results showed that the sorption process was not in agreement with both the Langmuir and the Freundlich models. Only nickel and chromium (VI) adsorption in both industrial and domestic polysaccharides fit the Langmuir plots since the square of correlation coefficient for the reciprocal plots was greater than 0.90 in both cases. Nickel adsorption by industrial sludge EPS fit the Freundlich plot (Fig. 5.31).

The rest of the isotherms showed high deviations from the straight line and confirm conformation to the third common model (Brunnauer-Emmett-Teller) describing the formation of more than one layer on the adsorption surface.

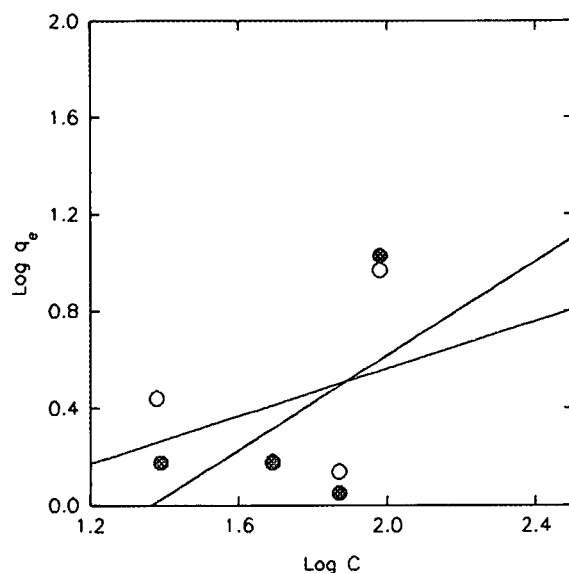


FIGURE 5.28. Freundlich linearised model for cadmium adsorption to domestic (○) and industrial (●) polysaccharides. Values given are means of three replicate experiments. Square of correlation coefficient for linearisation was 0.31 for industrial and 0.11 for domestic sludge EPS.

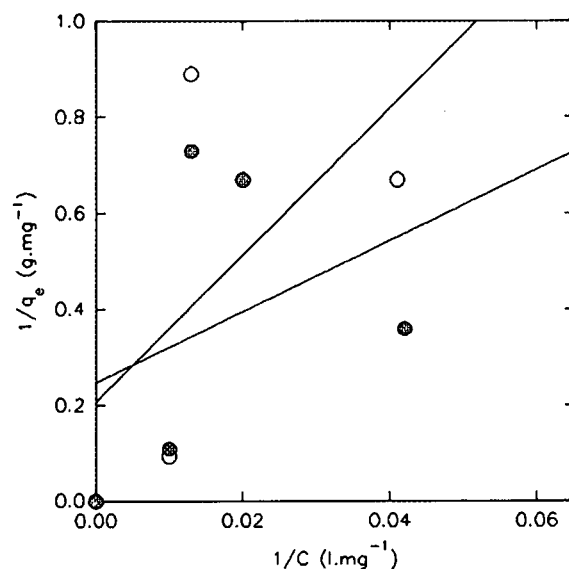


FIGURE 5.29. Freundlich linearised model for cadmium adsorption to domestic (○) and industrial (●) polysaccharides. Values given are means of three replicate experiments. Square of correlation coefficient for linearisation was 0.01 for industrial sludge EPS and 0.28 for domestic sludge EPS.

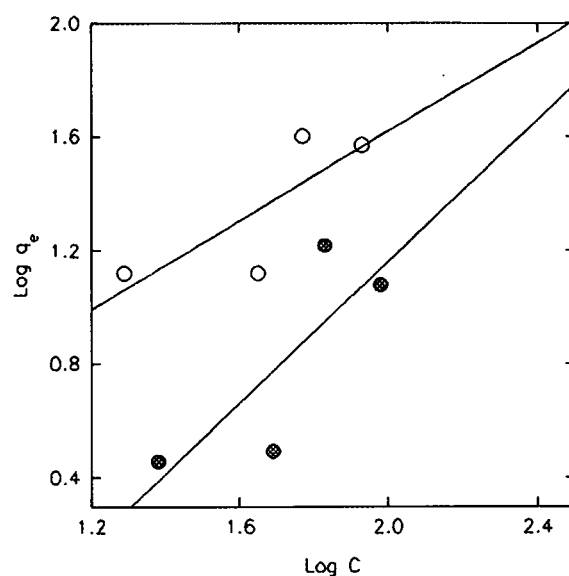


FIGURE 5.30. Freundlich linearised model for copper adsorption to domestic (○) and industrial (●) polysaccharides. Values given are means of three replicate experiments. Square of correlation coefficient for linearisation was 0.66 for industrial sludge EPS and 0.63 for domestic sludge EPS.

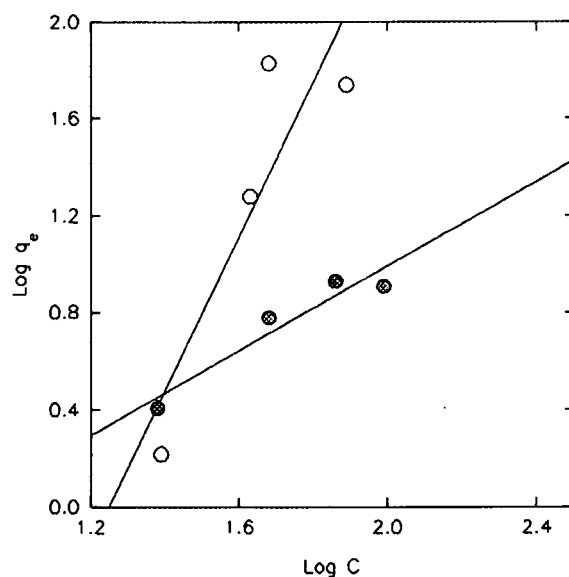


FIGURE 5.31. Freundlich linearised model for nickel adsorption to domestic (○) and industrial (●) polysaccharides. Values given are means of three replicate experiments. Square of correlation coefficient for linearisation was 0.91 for industrial sludge EPS and 0.78 for domestic sludge EPS.

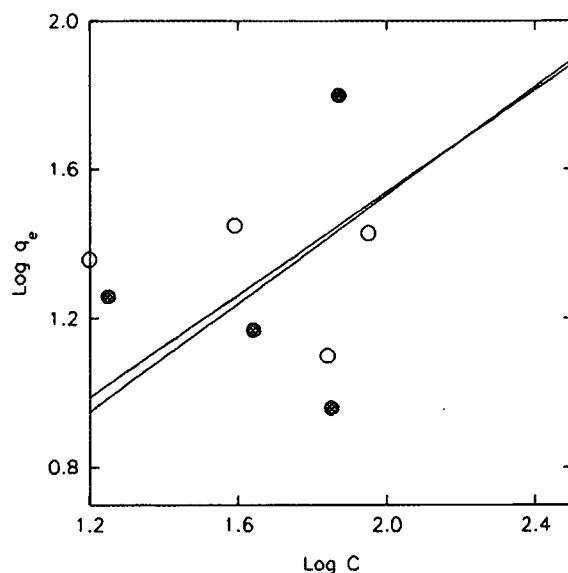


FIGURE 5.32. Freundlich linearised model for chromium (III) adsorption to domestic (○) and industrial (●) polysaccharides. Values given are means of three replicate experiments. Square of correlation coefficient for linearisation was 0.04 for industrial sludge EPS and 0.05 for domestic sludge EPS.

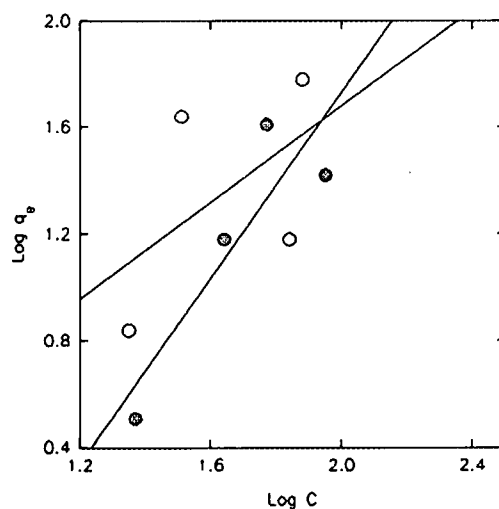


FIGURE 5.33. Freundlich linearised model for chromium (VI) adsorption to domestic (○) and industrial (●) polysaccharides. Values given are means of three replicate experiments. Square of correlation coefficient for linearisation was 0.78 for industrial sludge EPS and 0.29 for domestic sludge EPS.

5.6.4 Biosorption in a Ni^{2+} - Cr^{6+} mixture

The results of nickel and chromium (VI) biosorption as shown in the Langmuir plots necessitated the comparison of both these metals when added singly or in combination to polysaccharide samples in order to investigate any competitive or synergistic interactions which may influence metal removal.

Langmuir plots indicated that at low metal concentration complexation was controlled by similar binding sites. Therefore, further biosorptive studies had to be conducted to determine whether there were one or two groups of binding sites present. The choice of metals was based on the similarities of their Langmuir plots.

The experimental data series for the binary systems of Ni^{2+} - Cr^{6+} for domestic and industrial EPS are plotted in Figs 5.34 to Figs. 5.41. The effect on Ni^{2+} and Cr^{6+} removal due to the presence of competing ions was observed by comparing the two series. Adsorption of metals by EPS was approximated by a Langmuir-type adsorption isotherm model:

$$q = (bC_f q_{\max}) / (1 + bC_f)$$

where q_{\max} is the maximum metal uptake and b is the Langmuir constant, a ratio of the adsorption rate constant to the

desorption rate constant. The apparent dissociation constant for the sorption system (K) is the inverse of the Langmuir constant b.

Equation 1 can be converted into the following straight line equation:

$$\frac{1}{q_{\max}} = \frac{1}{bq_{\max}} \cdot \frac{1}{C_f} + \frac{1}{q_{\max}}$$

where $1/bq_{\max}$ is the slope and $1/q_{\max}$ is the constant.

Comparison of chromium (VI) adsorption in single solution and in a binary mixture indicates that large amounts of this metal are removed from a binary system (Figs. 5.36 and 5.37). Percentage adsorption by industrial polysaccharides of chromium (VI) did not show any trend (Figs. 5.36) but nickel adsorption determined as a percentage reached an optimal level at $50 \text{ mg} \cdot \ell^{-1}$ of nickel in solution. This is the opposite of what is observed in a single metal system where nickel adsorption is shown to have a continuous increase in adsorption and reaches an optimal level at $75 \text{ mg} \cdot \ell^{-1}$. Chromium (VI) adsorption reached an optimum level at $50 \text{ mg} \cdot \ell^{-1}$ in a binary mixture compared to $75 \text{ mg} \cdot \ell^{-1}$ in a single solution.

In general, metal biosorption in a binary mixture indicated that the presence of another metal in the mixture does not lower the sorption capacity for the other metal. Compared to single metal sorption, the total amount of metal biosorbed was higher when EPS was exposed to Ni-Cr mixture.

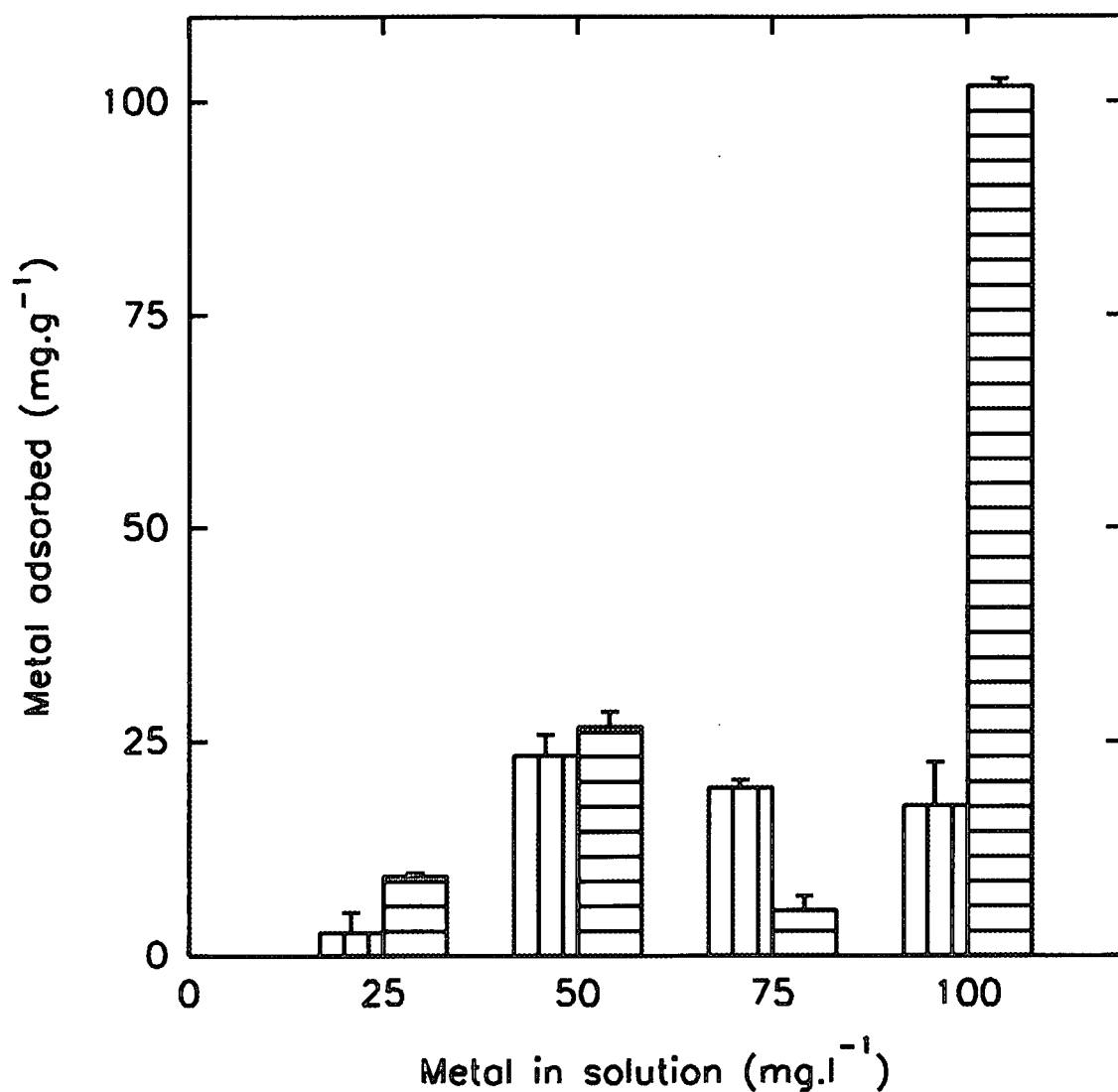


FIGURE 5.34. Comparison of the quantities of nickel (▨) and chromium (▤) adsorbed by industrial sludge polysaccharide in a Ni^{2+} - Cr^{6+} mixture at various metal concentrations. Histograms represent mean values of triplicates. Error bars represent deviation of the results from the mean.

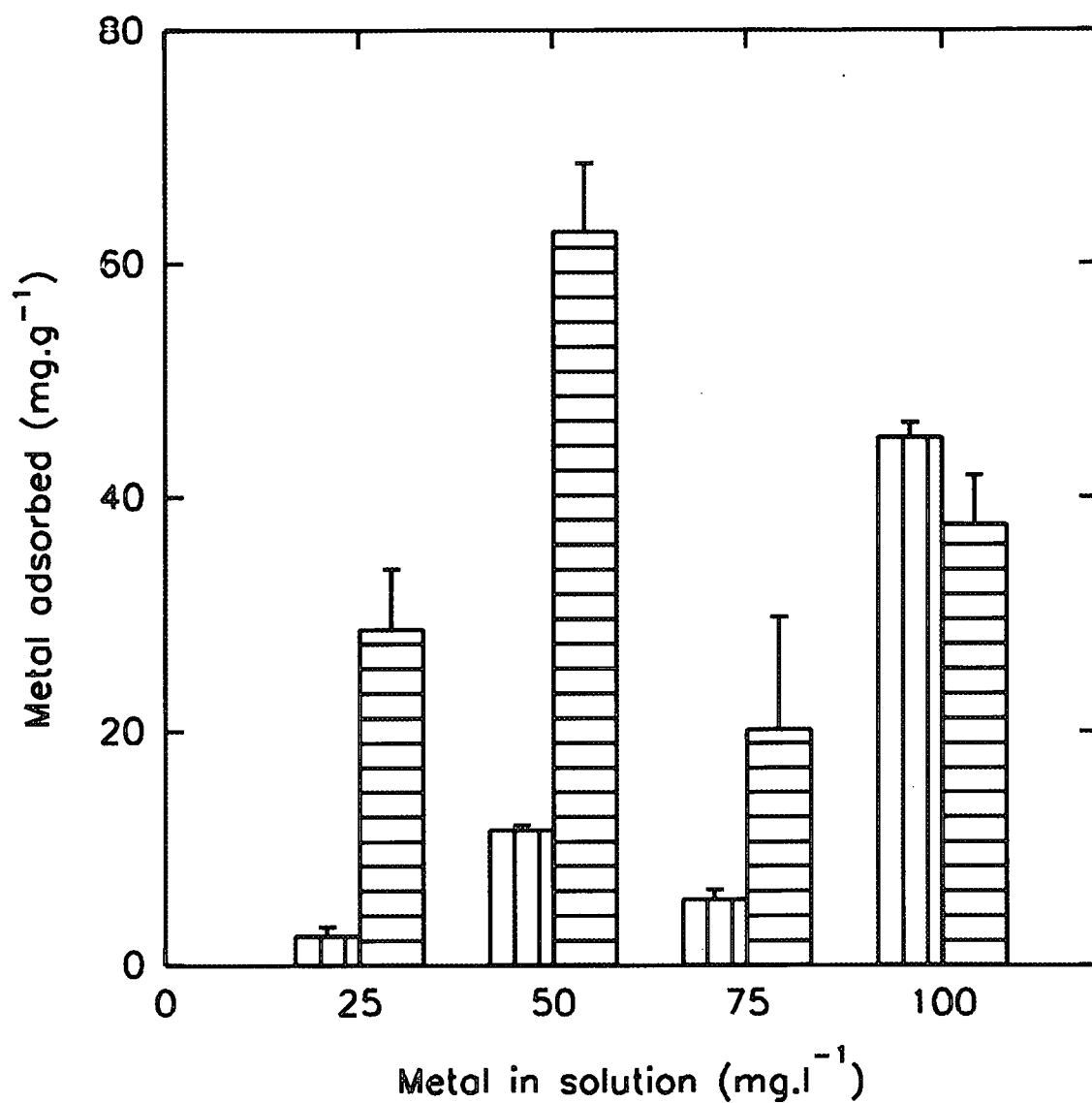


FIGURE 5.35. Comparison of the quantities of nickel (▨▨▨) and chromium (▨▨▨) adsorbed by domestic sludge polysaccharide in a Ni^{2+} - Cr^{6+} mixture at various metal concentrations. Error bars represent deviation of the results from the mean.

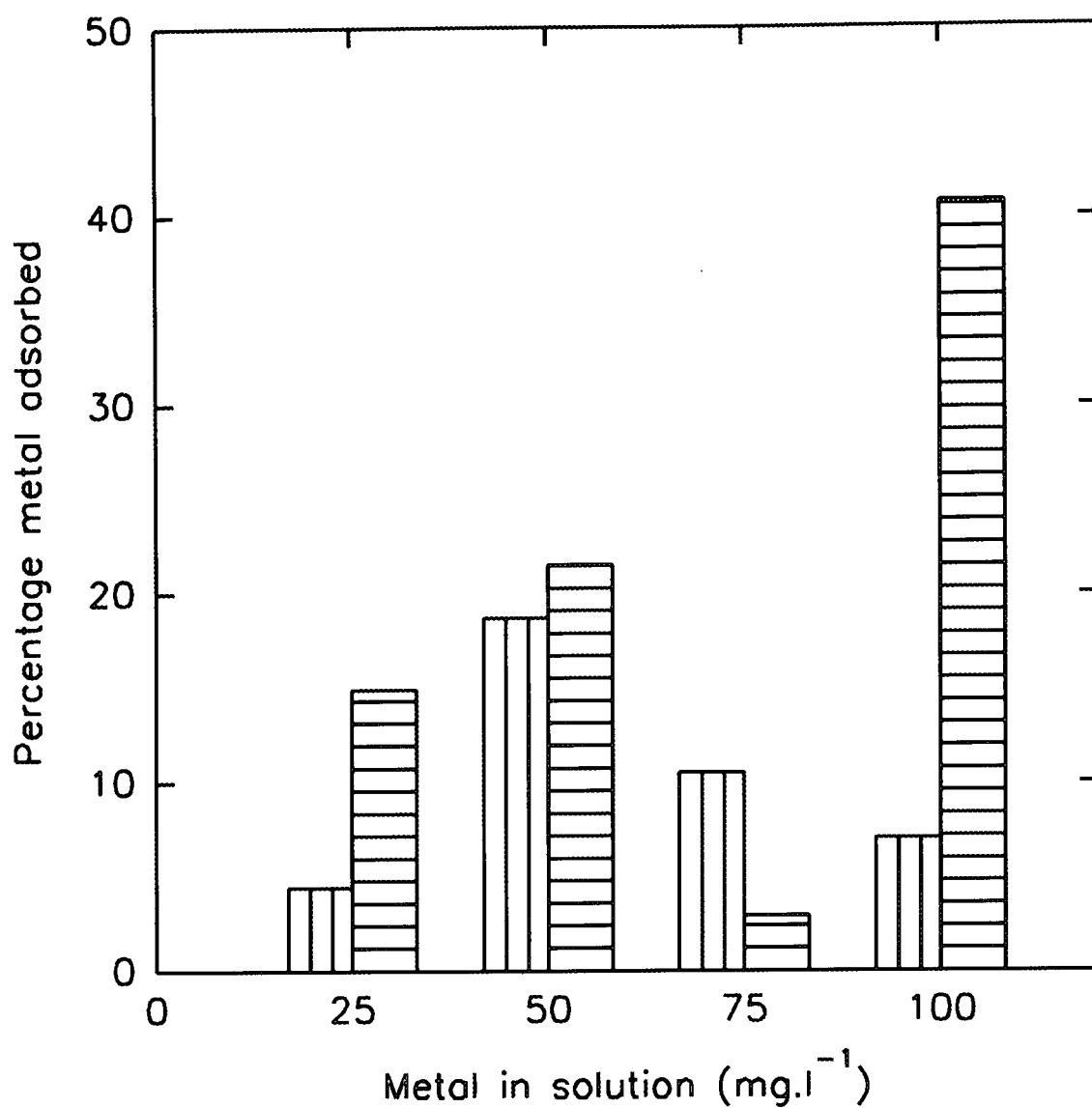


FIGURE 5.36. Comparison of the percentage adsorption of nickel (▨) and chromium (▤) by industrial sludge polysaccharide in a Ni^{2+} - Cr^{6+} mixture at various metal concentrations.

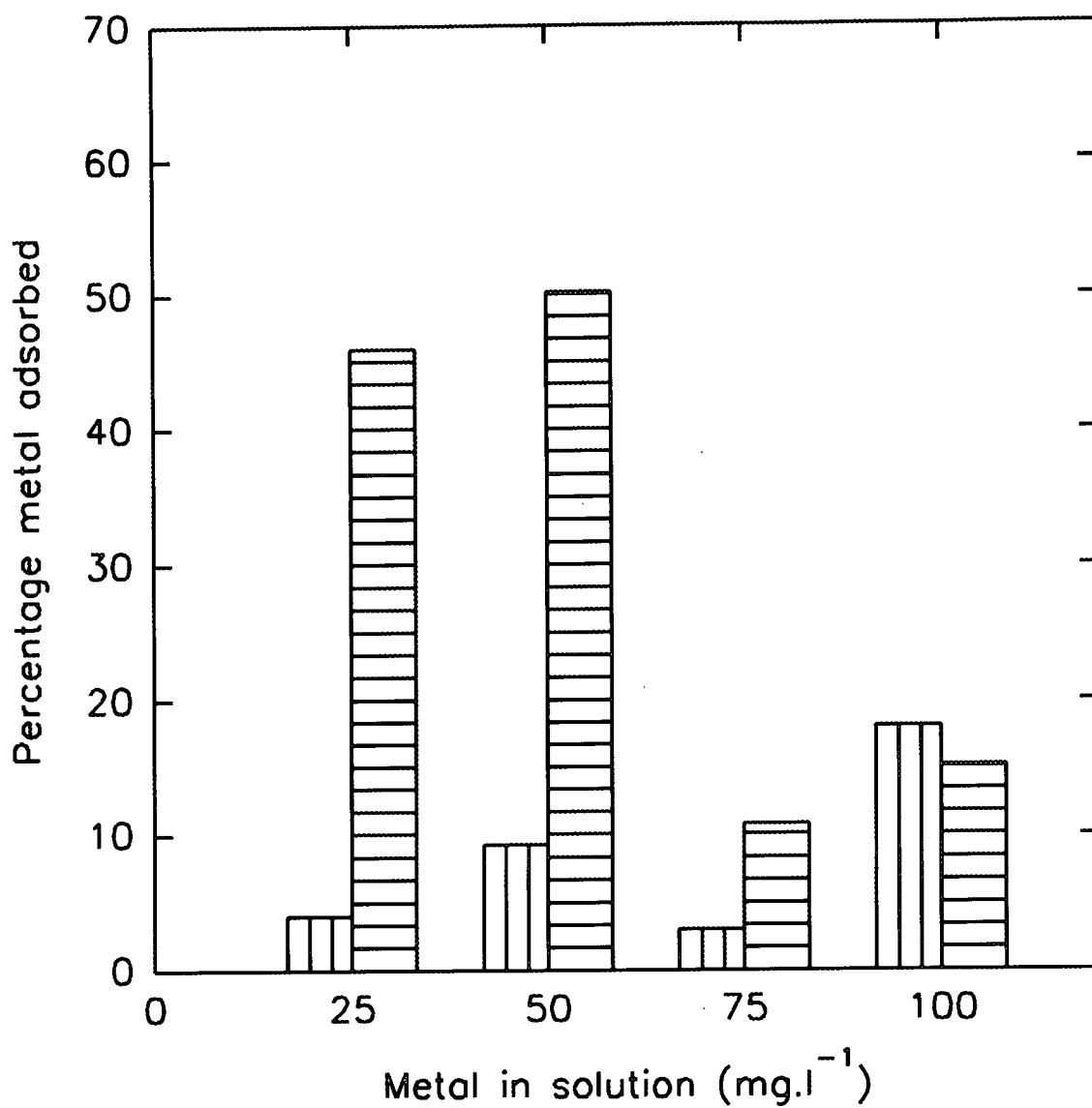


FIGURE 5.37. Comparison of the percentage adsorption of nickel (▨) and chromium (▤) by industrial sludge polysaccharide in a Ni^{2+} - Cr^{6+} mixture at various metal concentrations.

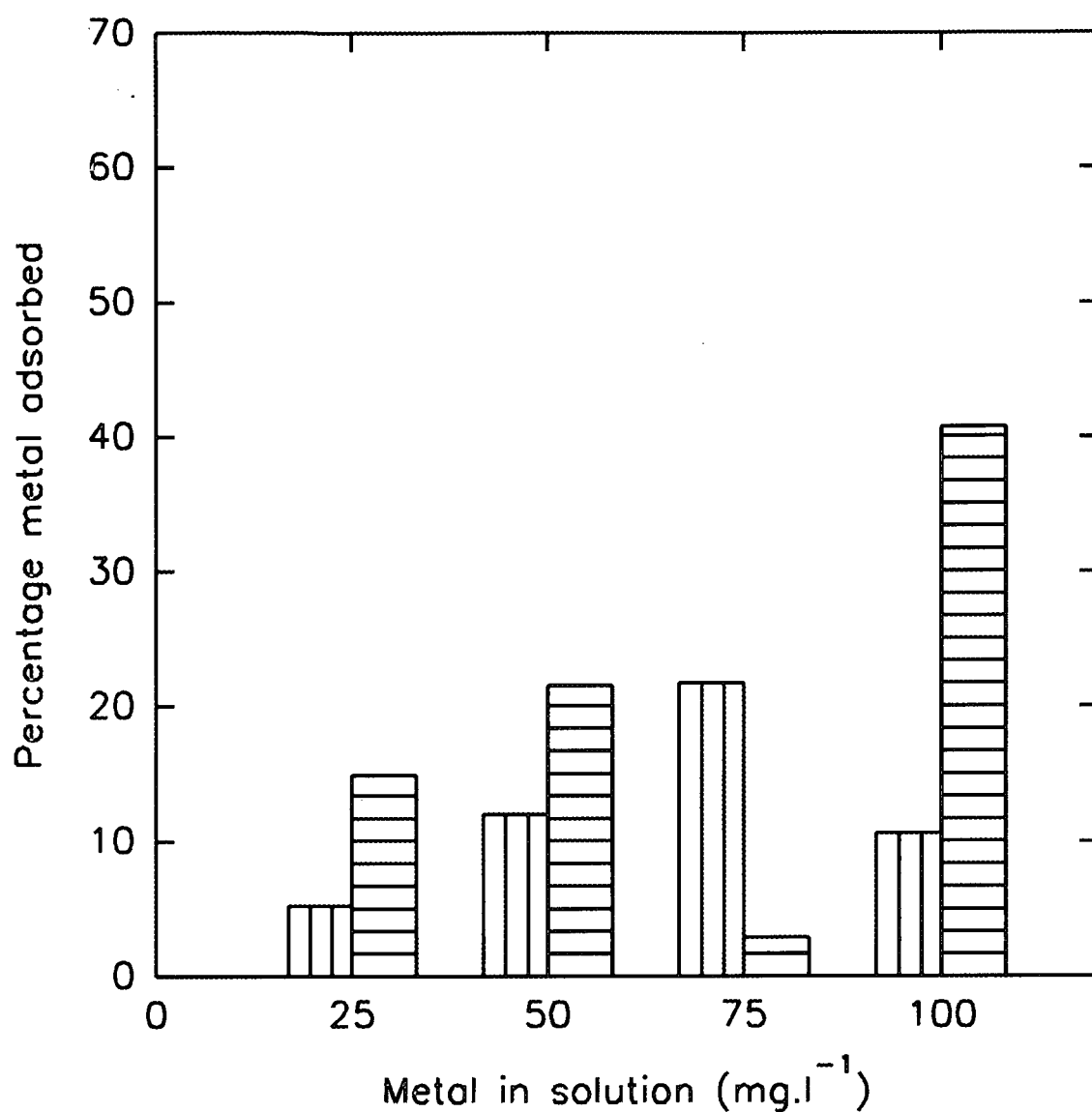


FIGURE 5.38. Comparison of the percentage adsorption of nickel (▮) and chromium (▮) by domestic sludge polysaccharide in a Ni^{2+} - Cr^{6+} mixture at various metal concentrations.

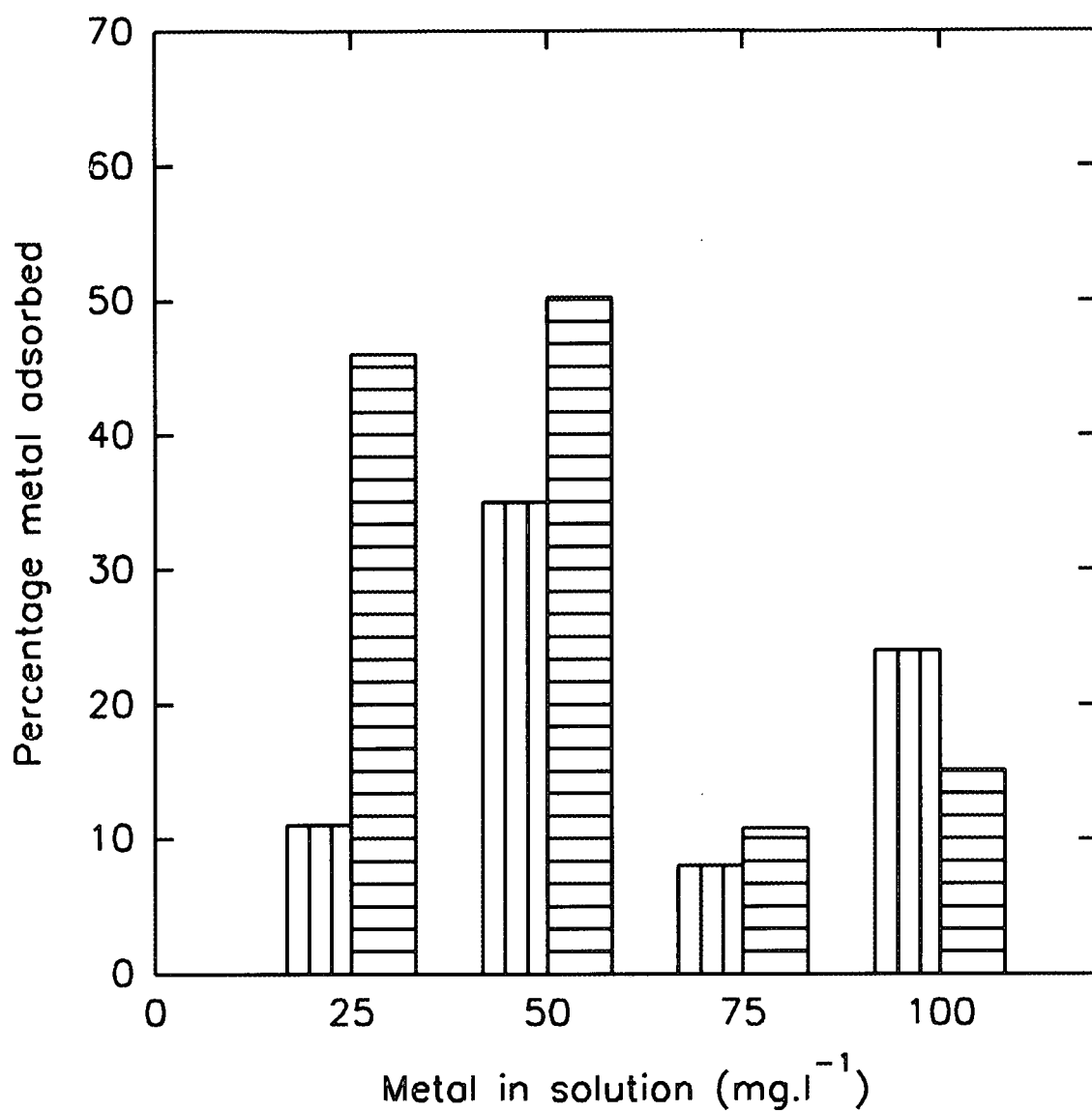


FIGURE 5.39. Comparison of the percentage adsorption of chromium in a single solution (▤) and in a Ni²⁺-Cr⁶⁺ mixture (▥) by industrial sludge polysaccharide at various metal concentrations.

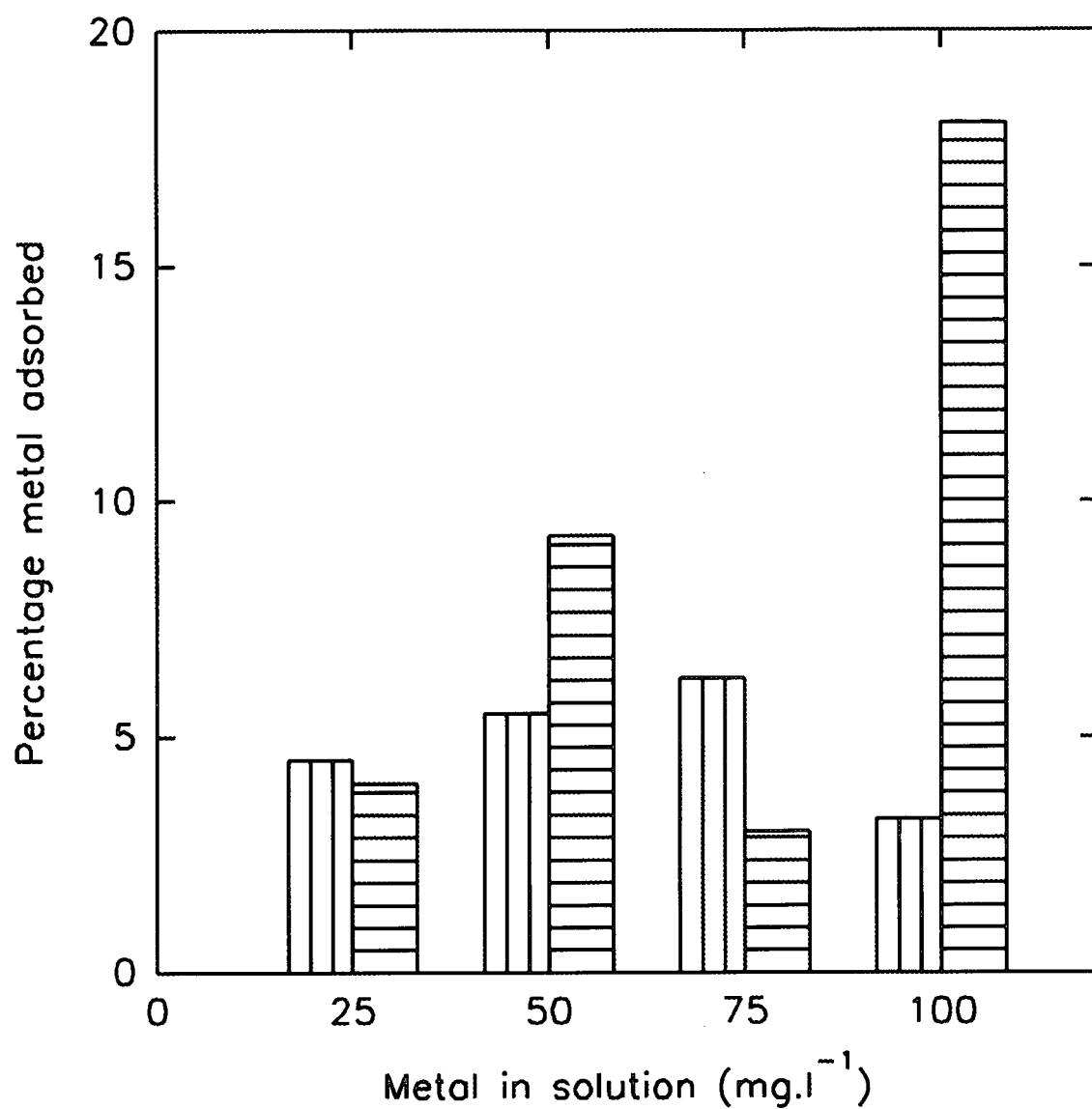


FIGURE 5.40. Comparison of the percentage adsorption of chromium in a single solution (▨) and in a Ni^{2+} - Cr^{6+} mixture (▤) by domestic sludge polysaccharide at various metal concentrations.

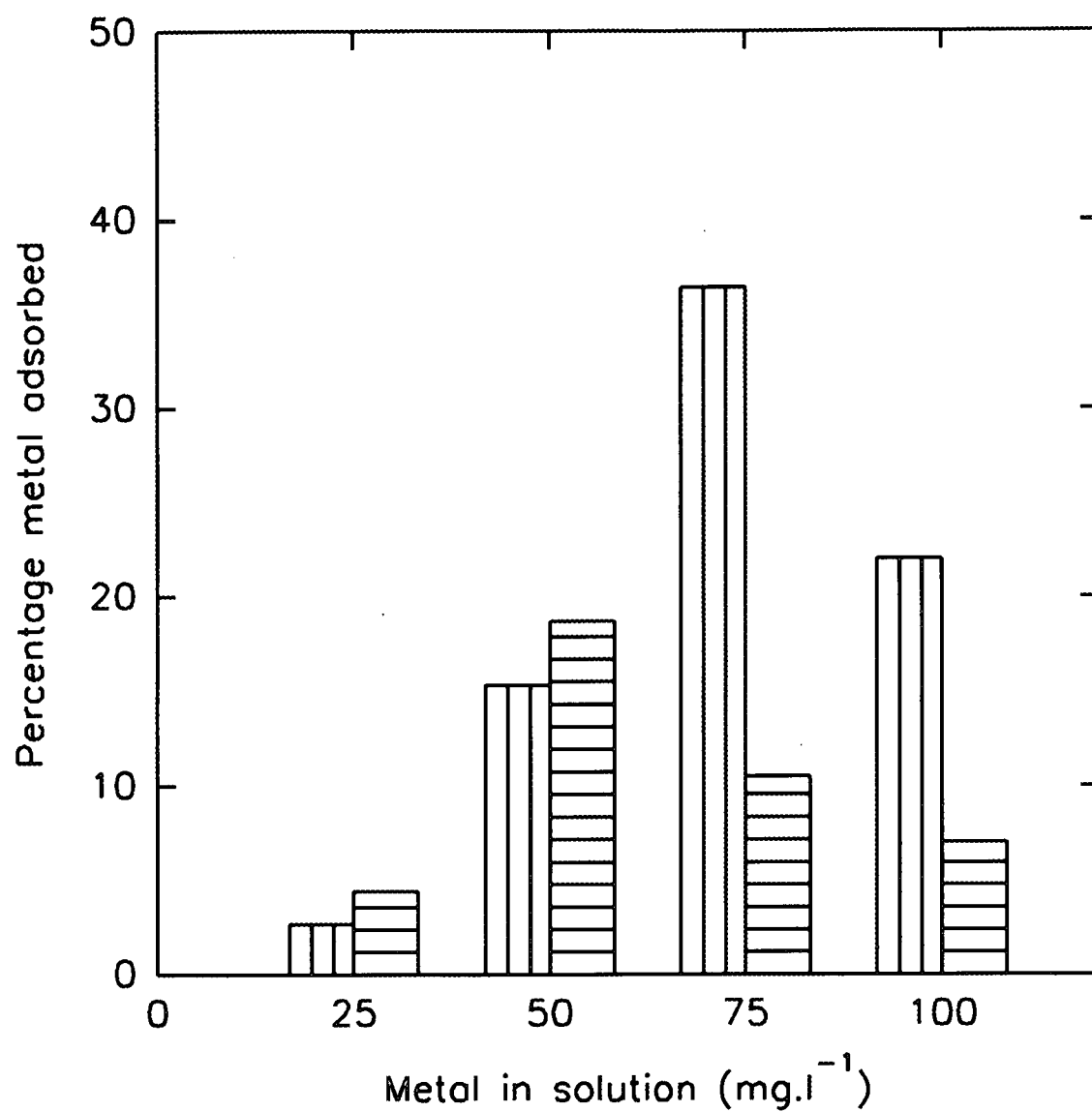


FIGURE 5.41. Comparison of the percentage adsorption of nickel in a single solution (■) and in a Ni^{2+} - Cr^{6+} mixture (▨) by industrial sludge polysaccharide at various metal concentrations.

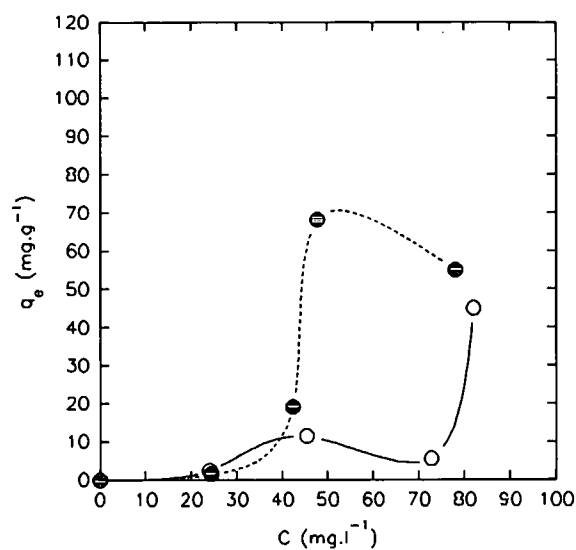


FIGURE 5.42. Langmuir adsorption isotherm of Ni^{2+} uptake from a $\text{Ni}^{2+}\text{-Cr}^{6+}$ mixture (○) and a single solution (●) by domestic sludge polysaccharides at varying equilibrium concentration C .

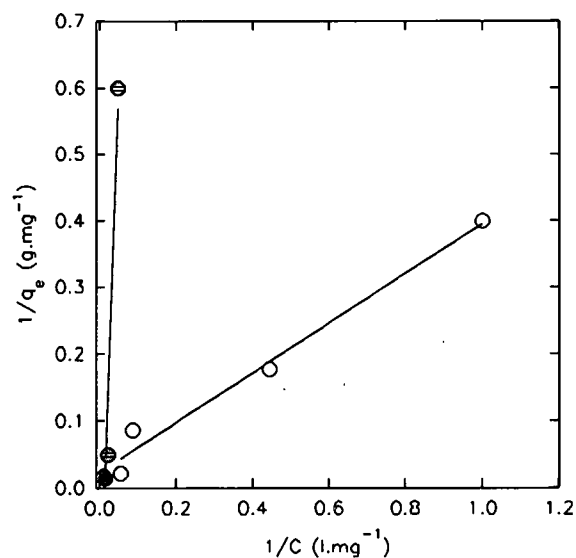


FIGURE 5.43. Reciprocal Langmuir plots for Ni^{2+} adsorption from a $\text{Ni}^{2+}\text{-Cr}^{6+}$ mixture (○) and a single solution (●) by domestic sludge polysaccharides at varying equilibrium concentration C .

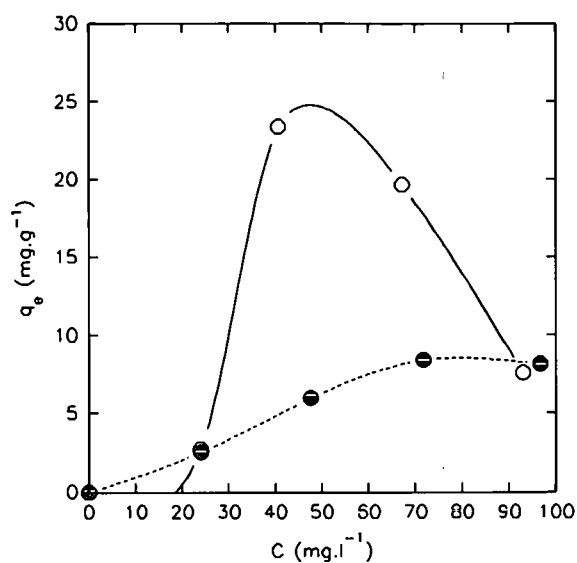


FIGURE 5.44. Langmuir adsorption isotherm of Ni^{2+} uptake from a Ni^{2+} - Cr^{6+} mixture (○) and a single solution (●) by industrial sludge polysaccharides at varying equilibrium concentration C .

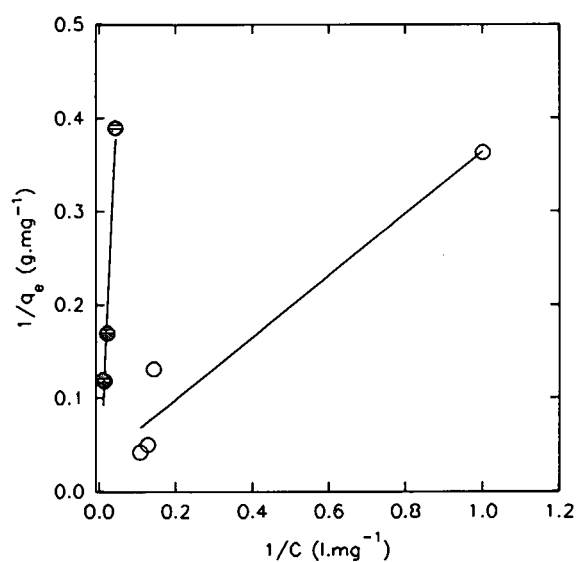


FIGURE 5.45. Reciprocal Langmuir plots for Ni^{2+} adsorption from a Ni^{2+} - Cr^{6+} mixture (○) and a single solution (●) by industrial sludge polysaccharides at varying equilibrium concentration C .

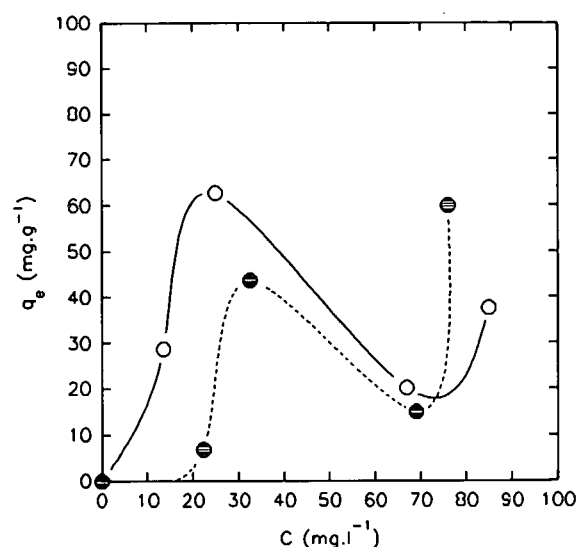


FIGURE 5.46. Langmuir adsorption isotherm of Cr^{6+} uptake from a Ni^{2+} - Cr^{6+} mixture (○) and a single solution (●) by domestic sludge polysaccharides at varying equilibrium concentration C .

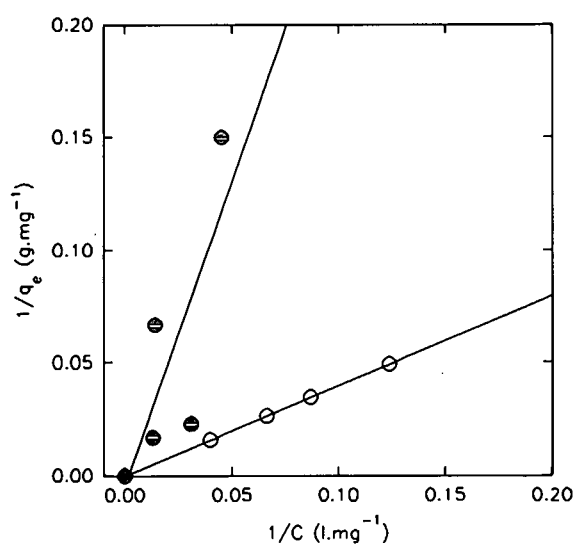


FIGURE 5.47. Reciprocal Langmuir plots for Cr^{6+} adsorption from a Ni^{2+} - Cr^{6+} mixture (○) and a single solution (●) by domestic sludge polysaccharides at varying equilibrium concentration C .

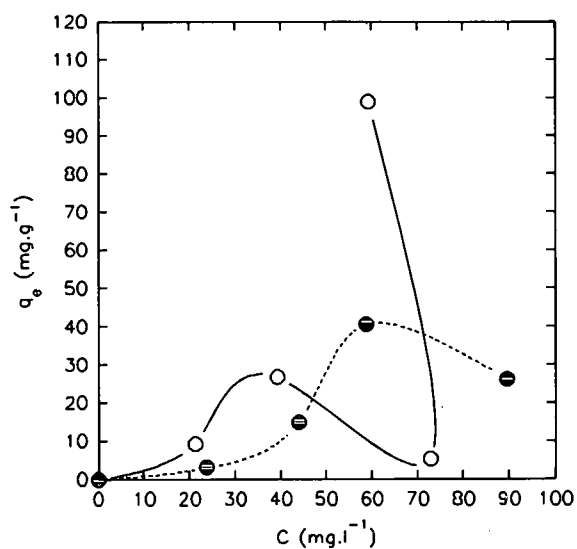


FIGURE 5.48 : Langmuir adsorption isotherm of Cr^{6+} uptake from a Ni^{2+} - Cr^{6+} mixture (○) and a single solution (●) by domestic sludge polysaccharides at varying equilibrium concentration C .

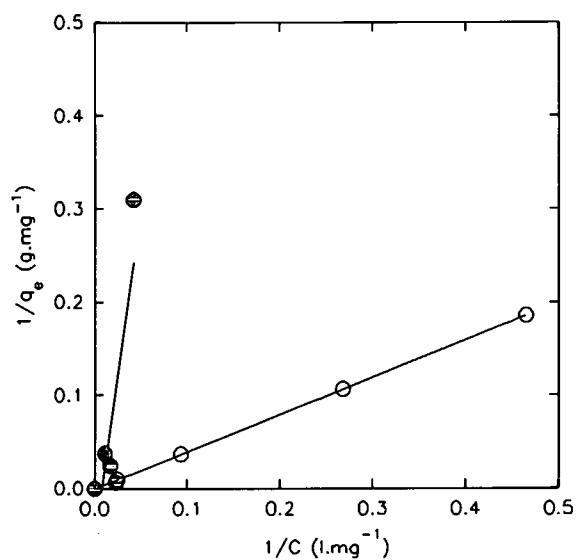


FIGURE 5.49 : Reciprocal Langmuir plots for Cr^{6+} adsorption from a Ni^{2+} - Cr^{6+} mixture (○) and a single solution (●) by domestic sludge polysaccharides at varying equilibrium concentration C .

5.7 DISCUSSION

Metal removal in the activated sludge process is an important phenomenon since it helps to reduce pollution of the receiving waters by heavy metals which are discharged into wastewater treatment plants. Many of the proposed mechanisms of metal removal involve extracellular polysaccharides. During the present study both domestic and industrial sludge EPS were capable of adsorbing the six metal species (Zn , Ni , Cu , Cr^{3+} , Cr^{6+} and Cd) from solution.

Selectivity of polysaccharides for a particular metal specie determined its ranking. Chromium was adsorbed with greater efficiency than all other metals while adsorption efficiency of nickel was greater than Cu , Zn and Cd in domestic EPS and less than Cu and Zn in industrial polysaccharides.

Overall, domestic sludge polysaccharides adsorbed larger average percentages of metal ions from solution than industrial sludge polysaccharides with the order of metal adsorption efficiency of: domestic sludge EPS : $\text{Cr}^{6+} > \text{Cr}^{3+} > \text{Ni}^{2+} > \text{Cu}^{2+} > \text{Zn}^{2+} > \text{Cd}^{2+}$, and industrial sludge EPS : $\text{Cr}^{3+} > \text{Cr}^{6+} > \text{Cu}^{2+} > \text{Zn}^{2+} > \text{Ni}^{2+} > \text{Cd}^{2+}$.

TABLE 5.2 Average percentage adsorption by domestic and industrial polysaccharides extracted from activated sludge.

Metal	Domestic EPS (%)	Industrial EPS (%)
Copper	17.0675	6.3500
Zinc	9.18500	5.9275
Nickel	19.0925	4.8775
Chromium (VI)	19.5000	12.355
Chromium (III)	19.1850	17.815
Cadmium	2.50750	2.1125

This confirms the study by Rudd, *et al.*, (1984) who calculated affinity series for sludge EPS adsorption to metal-ions to be Ni > Cu > Cd > Co > Mn > TI. Dugan and Pickrum (1972) determined metal affinity series to *Zoogloea ramigera* EPS to be Ni > Co > Cu which also confirms the results of the present study. With the exception of nickel, an increase in adsorption was observed as metal concentration increased whereas percentage adsorption decreased as metal-ion concentration in solution increased.

In the case of nickel, metal adsorption as well as percentage adsorption increased as metal concentration increased. This could be attributable to high quantity of proteins in both EPS samples. High amounts of proteins were found in activated sludge extracellular polysaccharide samples (Chapter 3).

This was confirmed by "L" shaped isotherms obtained for nickel which indicated that the binding is predominantly to carboxyl terminus residues which are contained in the protein fraction. "L" type adsorption isotherms have been proposed by Forster (1976) to be due to carboxyl groups of proteins. Glucuronic acid was also identified in both types of sludge and could be responsible for high metal biosorptive capability of sludge EPS. Hence, these components could be responsible for adsorption of nickel and chromium (VI) ions from solution. Gould and Genetelli (1984) reported substantial decrease in nickel adsorption when carboxyl sites on exopolymers were blocked.

The results of Langmuir isotherm graphs indicated that Zn^{2+} , Cr^{3+} , and Cd^{2+} displayed a "BET" relationship while Cr^{6+} and Ni^{2+} displayed "L-shaped" isotherms. This contradicts findings by Swalaha (1993) who observed "L" shaped isotherms for copper and "BET" type adsorption isotherm for chromium.

Langmuir model makes several assumptions such as monolayer adsorption and constant adsorption energy, while Freundlich equation deals with heterogenous adsorption. The agreement of the experimental data with both these models implies that both monolayer adsorption and constant adsorption energy existed for the experimental conditions used (Volesky, 1990)

Deviations from a straight line indicates that binding involves two or more classes of sites. Zn^{2+} , Cr^{3+} , and Cd^{2+} adsorption was mainly due to adsorption of these metal ions to hydroxyl groups of hexose sugars. Attachment is due to weak electrostatic forces originating from diffuse electron zones of one or more closely related hydroxyl groups (Swalaha, 1993).

Polysaccharide adsorption ranking, with respect to metal ions, was determined to be greater for domestic EPS than for industrial EPS. Comparison of the percentage adsorption of the two metals in a single solution and in a binary mixture produced unexpected results. Both chromium and nickel were more adsorbed by industrial polysaccharides in a Ni^{2+} - Cr^{6+} mixture than when they are in single solutions (Figs. 5.38 and 5.39).

The adsorption of nickel by domestic polysaccharides was an exception where nickel adsorption was less in Ni^{2+} - Cr^{6+} mixture than in single solution.

5.8 CONCLUSIONS

Domestic and industrial sludge extracellular polysaccharides adsorbed zinc, chromium, nickel, copper and cadmium metal-ions with domestic sludge EPS adsorbing larger quantities of metals from solution. Isotherms of nickel and chromium (VI) adsorption by both sludge EPS's were "L" shaped indicating that these metal ions could be utilising similar adsorption sites. This fact was contradicted by the results obtained from a binary mixture. Nickel and chromium were shown to be utilising different sites and therefore had a synergistic effect to each other.

CHAPTER SIX

6.0 GENERAL DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

6.1 DISCUSSION

Both domestic and industrial sludge EPS were capable of adsorbing all the metals used albeit in different degrees of adsorption (Figs. 5.14 and 5.15). In general the quantities of metals adsorbed increased as concentration of metal ions in solution increased. Overall, domestic sludge EPS adsorbed larger average percentages of metal-ions than industrial sludge EPS. The order of metal adsorption efficiency was $\text{Cr}^{6+} > \text{Cr}^{3+} > \text{Ni} > \text{Cu} > \text{Zn} > \text{Cd}$ for domestic sludge EPS and $\text{Cr}^{3+} > \text{Cr}^{6+} > \text{Cu} > \text{Zn} > \text{Ni} > \text{Cd}$ for industrial sludge EPS.

Components of extracellular polysaccharides such as glucuronic acids, thought to be responsible for adsorption, were found to be more abundant in domestic sludge polysaccharides. Due to the high protein content in domestic sludge EPS the degree of purity was found to be high in the industrial EPS (Table 3.2). Monosaccharides identified by paper chromatography in both polysaccharides samples were glucose, fructose, glucuronic acid and

galactosamine (Table 4.1 to 4.5). Any of these components may be responsible for high adsorption of metals by domestic sludge EPS. Glucose, glucuronic acid and galactosamine have also been identified by Horan and Eccles (1986) in sludge EPS.

Metal ions tend to form bonds with functional groups containing electron-donating atoms. Four types of interactions between metals and exopolymers have been described by Steiner *et al.*, (1976). The two most important types were those that involve salt bridges with carboxyl groups on acidic polymers and those that involved weak electrostatic bonds with hydroxyl groups on neutral polymers.

Both domestic and industrial sludge extracellular polysaccharide fractions were shown to contain amino sugars or sugars with amine-linked functional groups. These nitrogen containing functional groups can react with some metals although the mechanism involved has not been determined. Carboxyl groups as components of the proteins present in EPS samples could also be available to interact with charged metal ions. Glucuronic acid identified by paper chromatography (Table 4.5) may also be the binding site for metal ions. Protein and glucuronic acid content were found to be high in domestic sludge EPS. This may be the cause of high metal biosorptive capability of domestic sludge EPS.

The exopolysaccharide component of microbial exopolymers usually possess a repeating sequence of monosaccharides from two to six

sugar subunits (Ikeda et al., 1982). Some polymers, however, possess blocks of repeating sugars in one segment of polymer that are different from those in other segments. Many bacterial capsular polymers are composed of acidic polysaccharides (Brown and Lester, 1979). These acidic properties are contributed primarily by free carboxyl groups of uronic acid subunits or pyruvylated sugars.

The anionic character of EPS in any of the above segments is conferred by carboxylic acid moiety present (Horan and Eccles, 1986). Most of the components identified by chromatographic techniques in the present study were either at neutral, acidic or alkaline pH ranges. At neutral to alkaline pH, the carboxylic groups are partially ionised and are available to interact with positively charged metal ions. The polysaccharides also contain an abundance of hydroxyl groups which may also interact with metal ions. The electronegative oxygen atoms of hydroxyl groups are likely to participate in metal interactions with anionic or neutral polysaccharides. The bond formed between carboxylic groups and metal ions is ionic and is much stronger than the hydrogen bonding between hydroxyl groups and metal ions (Kasan, 1993).

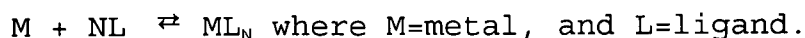
The evidence presented above suggests that different metal adsorption sites exist on neutral and anionic polysaccharides. Metal ions of different valencies or with different charges bind at different sites. Because some of the metals such as nickel and

chromium (VI) exhibited Langmuir isothermic behaviour on both types of sludge EPS, it can be concluded that the adsorbent groups were intrinsically homogeneous and that the surface has a specific number of nickel or chromium binding sites. Each of these sites are considered to be either the carboxyl groups or the hydroxyl groups and when these are all occupied no further adsorption is possible. These different metal adsorption sites can exist on neutral polysaccharides and anionic polysaccharides. Binding of metals to bacterial EPS has been reviewed by Sterrit and Lester (1980). They divide the uptake of metals into L- and S- shaped. The L-shaped type (expressed by a Langmuir isotherm) describes an adsorptive mechanism whereby as more metal is bound, there is a progressive decrease in the availability of binding sites, primarily exhibited by charged carboxyl groups (stoichiometry of -COOH to metal interaction is 1:1). With S-type curves, the presence of metals during the initial stages of adsorption promotes an increase in the quantities subsequently adsorbed, possibly due to the hydroxyl groups of hexose sugars (Steiner et al, 1976).

Dugan (1970) has reported that an extracellular polymer produced by *Z-ramigera* 1-16 bind metal ions to hexuronic acids which are present in the cell walls. He suggested that the metal binding sites for the "L" type isotherms are carboxyl groups. This suggests that the carboxyl groups on the EPS are already occupied as the concentration increases and reaches an optimum limit. Forster (1976) has expanded this theory by including the carboxyl

groups of proteins in producing the "L" type metals adsorption isotherms

Hughes and Poole (1989) postulated that the mechanism involved in the interactions between metals and ligands (polysaccharides) with equilibrium equation is:



When interactions such as these occur, stoichiometry constraints due to ligand positioning may restrain adsorption.

Since hexose and pentose monomers were found in the sludge exopolymers, Friedman *et al.*, (1986) proposed the adsorption sites for the "S" isotherms to be hydroxyl groups of the rings. This can be extrapolated to the present study where glucose and fructose were found to be monomers present in both types of polysaccharides. The attachment of metals to these EPS can be considered to be caused by weak electrostatic forces. Friedman *et al.*, (1986) proposed that as hydroxyl groups would be most certainly be hydrated, adsorption of the metal ion would result in a decrease in the bound water content to the polymer. This agrees with the work done earlier by Dugan *et al.*, (1969).

If this theory is correct, it suggests that in the activated sludge process, the "S" type adsorption isotherms of EPS to metal binding is the more important mechanism of metal removal. This confirms

our finding that, with the exception of nickel and chromium (VI) in domestic sludge EPS, chromium (III), zinc, copper and cadmium demonstrated an "S" type adsorption isotherm.

Rudd *et al.*, (1984) suggested that these binding sites are hydroxyl units of the hexose or pentose rings of neutral polysaccharides. Where polysaccharides are anionic polyelectrolytes carboxyl groups may be the metal binding sites. Forster (1985) suggested that the nickel ion is small and whilst it was shown to be unable, sterically, to form carboxyl complexes it can do so as carboxyl groups become more frequent.

The present results are compatible with an evaluation by Forster (1983) of the number of binding sites of exopolymer in relation to the size of the ion being bound which showed that more binding sites were available for larger ions than for smaller ions. Forster (1985) suggested that metal ions with different valencies may bind at different sites. The evidence presented here also confirms the Brown and Lester (1979) suggestion that different metal adsorption sites exist in neutral and anionic polysaccharides. Copper and zinc which are both large ions can form both hydroxyl and carboxyl complexes. The high adsorption of metals by domestic EPS which was shown to contain high protein and uronic acids content confirms Forster's (1985) theory that the principle ionogenic component of sludge polysaccharides is glucuronic acid.

Forster (1983) postulated that ionic radius determines the binding efficiency of metal-ions and stated that metal-ions with larger radii are capable of forming complexes with both carboxyl and hydroxyl groups whereas metals with smaller radii cannot form carboxyl-based complexes and this could result in lower biosorption of those metal-ions. Bonner and Smith (1957) discovered that ions with larger atomic radii were adsorbed more efficiently.

The ionic radii of Cd, Ni, Zn, Cu, Cr^{3+} and Cr^{6+} are 0.97, 0.80, 0.74, 0.72, 0.64 and 0.35 respectively (Forster, 1985). In terms of these ionic radii comparisons and the effect of ionic radii discussed earlier, the anomaly recorded during the present study was that metal ions with lower ionic radii were adsorbed more efficiently. The hypothesis which can be proposed is that for metal ions of equal charge, molecular size determines the order of preference of adsorption. The smaller ions are able to accomplish a closer approach to the adsorption site and thus have a favourable adsorption. Another reason for the low adsorption of metal ions with larger radii can be attributed to the low number of carboxyl binding sites compared to hydroxyl binding sites present in polysaccharides which are specific for metal ions with large radii.

It can then be proposed that nickel binds to carboxyl groups and chromium (VI) binds to hydroxyl groups since it is the smallest of the metal ions and as such unable to form carboxyl-based complexes. The ionic radii of nickel and chromium (VI) ions as well as the

fact that these metal ions produces L-shaped isotherms confirms that nickel and chromium binds to homogenous types of sites. The differences in the adsorption sites of nickel and chromium (VI) ions is confirmed by the comparison of both these metals when added in combination to EPS samples. The results indicated a synergistic rather than a competitive interactions that results in an increase in adsorption of these metals in a binary system.

The solution pH may have affected the solution chemistry of the metals, the activity of functional groups in the polysaccharides as well as the competition of metallic ions for the binding sites. At an alkaline pH the carboxylate groups of the polysaccharides may largely be dissociated generating negatively charged surfaces. Electrostatic interactions between cations species and the negatively charged surfaces may be responsible for the metal binding. Since the pH used for the present study was a neutral pH, the carboxylic groups were partially ionised and were able to interact with the positively charged ions.

It could be argued that the extracellular polysaccharide produced as a solid in the laboratory is different from the native polymer in the sludge. Steiner *et al.*, (1976) suggested that any modification to the polymeric material brought about by extraction and purification procedure do not alter the nature of the binding sites. But the extrapolation of these results to metal/sludge behaviour should take into account the contribution of other

polymers to metal biosorption and it is considered to be unlikely that any one type of molecule or functional group is responsible for the adsorption of metals (Tobin et al., 1984).

6.2 CONCLUSIONS

- a) Higher amounts of EPS were isolated from domestic sludge than industrial sludge samples.
- b) Four monomers namely: glucose, fructose, galactosamine and glucuronic acid and three unidentified monomers are present in the sludge EPS.
- c) Glucuronic acid confer a negative charge which would not be present in neutral polysaccharides.
- d) EPS are capable of binding metal ions in solution.
- e) Binding of metals to binding sites is dependent on chemical composition of extracellular polysaccharides; monomer composition and ionic radii of metal ions.
- f) Nickel and chromium(VI) have a synergistic interaction when adsorbed in a Ni^{2+} - Cr^{6+} mixture.

6.3 RECOMMENDATIONS

- (a) Further elucidation of the extracellular polysaccharide's functional groups which are active in the metal-sequestering process is necessary.
- (b) The biochemical processes responsible for biosynthesis and formation of the extracellular polysaccharides needs to be understood.
- (c) EPS-producing microorganisms present in the activated sludge need to be identified.

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APPENDICES

APPENDIX A

The Folin-Lowry method of protein assay (Lowry *et al.*, 1957).

PRINCIPLE

Protein reacts with the Folin-Ciocalteu reagent to produce a coloured complex which is due to the reaction of the alkaline copper with the protein as in the biuret test and the reduction of phosphomolybdate by tyrosine and tryptophan present in the proteins.

MATERIALS

1. Alkaline sodium carbonate solution ($20 \text{ g.l}^{-1} \text{ Na}_2\text{CO}_3$ in 0.1 mol/l NaOH).
2. Copper sulphate-sodium potassium tartrate solution ($5\text{g/l CuSO}_4 \cdot 5\text{H}_2\text{O}$ in $10\text{g/l Na, K, tartrate}$). Prepare fresh by mixing stock solutions.
3. 'Alkaline solution'. Prepare on day of use by mixing 50 ml of (1) and 1 ml of (2).
4. Folin-Ciocalteu reagent. (Dilute the commercial reagent with

an equal volume of water on the day of use. This is a solution of sodium tungstate and sodium molybdate in phosphoric and hydrochloric acid).

5. Standard protein (albumin solution 0.2 mg/ml).

METHOD

Add 5 ml of the 'alkaline solution' to 1 ml of the solution. Mix thoroughly and allow to stand at room temperature for 10 min or longer. Add 0.5 ml of diluted Folin-Ciocalteu reagent rapidly with immediate mixing. After 30 min read the extinction against the appropriate blank at 750 nm.

APPENDIX B

Determination of total sugars in EPS extracts (Dubois *et al.*, 1958).

Reagents

1. Sulphuric acid 95.5 %
2. Phenol 80 % (w/v)

Standard concentration of glucose in deionised water to concentration of 1, 2, 5, 10, 20, 50, 100 and 200 mg/l. Deionised water comprised blanks.

Methodology

1. Add 2 ml of blanks, standards and test solutions in triplicate into 20 ml test tubes.
2. Pipette 0.05 ml 80% phenol into sugar solutions.
3. Add 5 ml sulphuric acid directly into liquid in test tubes with a rapid delivery pipette
4. Allow to stand for 10 min., then mix in a vortex mixer.
5. Place in a 25 °C water for 10-20 min.
6. Read absorbances of standards and samples at 490 nm.

APPENDIX C

Determination of amino sugars glucosamine and galactosamine in purified EPS from domestic and industrial sludges (Rondle and Morgan, 1955).

Reagents

A- Acetylacetone reagent (1 ml Acetylacetone dissolved in 0.5 N Na_2CO_3 solution).

B- Ethanol (absolute).

C- p-Dimethylaminobenzaldehyde reagent (0.8 g aldehyde dissolved in 30 ml conc. HCl stored at 10 °C).

1% (W/V) Phenolphthalien, 1N NaOH, 0.3N HCl. Standard concentrations of glucosamine and galactosamine at 1, 5, 10, 20, 50 and 100 mg/l. Deionised water comprised blanks.

Methodology

1. Add 1 ml blank, standard and test solution into each of three 20 ml test tube graduated at 3 and 10 ml in triplicate.
2. Add 1 ml reagent A to test tubes and wash walls with 1 ml deionised water.

3. If test solution is acidic add 1 drop phenolphthalein before acetylacetone and a small quantity of NaOH to achieve full pink colour. Dropwise, add 0.3N HCl until colourless, bring up to 3 ml with deionised water. Lightly stopper tubes with glass freeze-drying vials sealed with 2 ml deionised water to act as condensers and boil for 20 min., in water bath.
4. Cool to room temperature and add 5 ml reagent B and 1 ml reagent C and bring to 10 ml with deionised water and mix.
5. Warm to 65-70 °C for 10 min., then cool to 18 °C.
6. Read absorbance of samples at 530 nm.

APPENDIX D

Determine of hexuronic acids, glucuronate and galacturonate (Dische, 1947) in purified ECP from domestic and industrial sludges.

Reagent

A- Conc. H_2SO_4

B- 0.1% Carbazole (w/v) in absolute ethanol

Standards of glucuronic and galacturonic acids at concentrations of 5, 10, 50 and 100 mg/l. Deionised water comprised blanks.

Methodology

1. 1 ml of blank, standards and samples is mixed in triplicate with 6 ml reagent A in 20 ml test tubes in a water bath.
2. Solution is heated for 20 min., in a boiling water bath.
3. Add 0.2 ml reagent B with shaking and allow to stand for two hours.
4. Read absorbance of samples at 530 nm within 1 hour.

APPENDIX E

Periodate oxidation (Stenesh, 1972)

Reagent

1. Methyl- D-glucoside
2. Amylopectin or glycogen
3. 0.4M NaIO_4
4. 0.5M H_2SO_4
5. 10% NaI
6. 0.1M $\text{Na}_2\text{S}_2\text{O}_3$
7. 1% EPS (mg/l)
8. 1% Phenolphthalein indicator
9. 0.05M NaOH
10. Ethylene glycol

Procedure

1. Weigh out 0.200 g of polysaccharide (glycogen or amylopectin) to the nearest mg and dissolve it in about 20ml of water. Boil vigorously to dissolve the polysaccharide, then cool and transfer to the solution quantitatively to a second 50 ml volumetric

flask.

2. Add 25 ml of 0.4 M NaIO_4 to each of the two volumetric flasks and make up to volume with distilled water.
3. Prepare a blank by adding 25 ml of 0.4 M NaIO_4 to a third 50 ml volumetric flask diluting to volume with distilled water.
4. Incubate all 3 volumetric flasks in the dark in your locker for 48 hours.

B. Titration

5. Just prior to the incubation period prepare a 125 ml conical flask containing 10 ml of a 0.5 M H_2SO_4 and 10 ml of 10% NaI .
6. At the end of the incubation period, remove a 1.0 ml aliquot from the volumetric flask containing the blank (step 4) and add it to the conical flask of step 6.
7. Immediately titrate the I_2 released with standard 0.1 M $\text{Na}_2\text{S}_2\text{O}_3$. Do not add the $\text{Na}_2\text{S}_2\text{O}_3$ too fast.
8. When the colour of the solution is a pale yellow, add 1.0 ml of 1% starch solution.
9. Continue titrating with $\text{Na}_2\text{S}_2\text{O}_3$ until the blue colour (due to the starch iodine complex) just disappears.
10. Repeat steps 6 to 11 for the conical flask containing the polysaccharide reaction mixture. (step 3).
11. After the $\text{Na}_2\text{S}_2\text{O}_3$ titration, remove two 10 ml aliquots

from each of the three volumetric flasks and add each aliquot to 125 ml conical flask containing 2 ml of ethylene glycol. Mix.

12. Allow the conical flasks to stand at room temperature in the dark for the 15 minutes with occasional swirling. The ethylene glycol reduces excess periodate.
13. Add a drop of 1% phenolphthalein indicator to each conical flask and titrate the solution with a standardized. CO_2 -free solution of 0.05 M NaOH (store the 0.05 M NaOH in a polyethylene bottle, equipped with a ascarite tube to exclude carbon dioxide).
14. Titrate all 6 samples to a pink phenolphthalein end point.
15. Compute the average titrant volume for each set of duplicate samples.